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BACTERIAL INFECTIONS IN TYPE 1 DIABETES AND THEIR ASSOCIATION WITH MICRO- AND MACROVASCULAR COMPLICATIONS

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ACADEMIC DISSERTATION

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"*People think of education as something they can finish*.

The true delight is in the finding out rather than in the knowing."

— Isaac Asimov

ABSTRACT

Background. Individuals with diabetes are more susceptible to bacterial infections compared with the general population. In individuals without diabetes, these infections have been associated with micro- and macrovascular diseases, such as chronic kidney disease and cardiovascular disease. However, the role of bacterial infections in the aetiology of these diseases is unclear, and may be profound in individuals with diabetes, who suffer from both bacterial infections as well as micro- and macrovascular disease more frequently compared to the general population. Furthermore, the prevalence of bacterial infections in individuals with specifically type 1 diabetes and the impact of hyperglycaemia on infection frequency is also far from established. Finally, the potential genetic factors affecting infection susceptibility in diabetes are yet to be discovered.

Aim. The aim of this thesis was to investigate the frequency of bacterial infections in individuals with type 1 diabetes and how the infections associate with and potentially affect the risk of developing diabetic kidney disease, coronary heart disease, and diabetic retinopathy. Moreover, we investigated whether common variations in the genome were associated with the susceptibility to bacterial infections observed in diabetes.

Methods. The studies presented in this thesis were conducted within the national multicentre study FinnDiane (Finnish Diabetic Nephropathy Study Group). The FinnDiane cohort consists of individuals with type 1 diabetes, recruited from all over Finland as well as non-diabetic control subjects from the general population. For all individuals included in the studies, data on bacterial infections treated both outside and within hospitals were collected from two nationwide registries: The national Finnish Hospital Discharge Register (Finnish Care Register for Health Care, HILMO) and the Finnish National Drug Prescription Register (KELA). Data on the emergence or progression of chronic diabetic complications as well as relevant clinical risk factors were collected during baseline and prospective clinical study visits, as well as from medical files collected from primary health care centres and hospitals across the country. Genomic DNA was extracted from blood leukocytes and bacterial lipopolysaccharide (LPS) activity was determined from serum samples during the baseline visit.

Results. Bacterial infections were found to be roughly two times more common in individuals with type 1 diabetes, compared to non-diabetic control subjects. Infections were more frequent in individuals with diabetic kidney disease and/or poor glucose control. Frequent antibiotic purchases and high LPS-activity were found to be independent risk factors for incident coronary heart disease as well as severe diabetic retinopathy in type 1 diabetes. Genome-wide association studies (GWAS) on individuals with diabetes revealed a potential association between variants on chromosome 2 and a reduced infection susceptibility. This association between the genetic loci and infection frequency was possibly mediated through the regulation of the *IRAK1*-pathway.

Conclusion. Bacterial infections are more frequent in individuals with type 1 diabetes than in the general population. Frequent antibiotic purchases and high levels of LPS-activity associate with the development of both micro- and macrovascular complications. Genetic factors on chromosome 2 may further influence the susceptibility to bacterial infections present in diabetes.

TIIVISTELMÄ

Tausta. Diabetesta sairastavilla henkilöillä on taustaväestöön verrattuna suurempi riski sairastua bakteeriperäisiin infektioihin. Taustaväestössä nämä infektiot ovat usein liitetty mikro- ja makrovaskulaaritauteihin (mm. munuaistauti, sydän- ja verisuonitaudit) mutta infektioiden merkitys näiden tautien etiologiassa on epäselvää. Infektioiden merkitys korostuu erityisesti diabetesta sairastavilla henkilöillä, jotka kärsivät sekä bakteeri-infektioista että mikro- ja makrovaskulaaritaudeista muuhun väestöön verrattuna useammin. Nykytietämys erityisesti tyypin 1 diabetesta sairastavien henkilöiden infektioiden esiintyvyydestä sekä hyperglykemian vaikutuksesta infektioriskiin on ollut toistaiseksi puutteellisia. On myös huomattava, että diabetesta sairastavien henkilöiden infektioherkkyyteen vaikuttavat geneettiset riskitekijät ovat vielä löytämättä.

Tavoite. Väitöskirjan tavoitteena oli tutkia bakteeri-infektioiden esiintyvyyttä tyypin 1 diabetesta sairastavilla henkilöillä sekä selvittää miten infektiot vaikuttavat riskiin sairastua diabeettiseen munuaistautiin, sepelvaltimotautiin ja diabeettiseen retinopatiaan. Lisäksi selvitimme infektioherkkyyteen vaikuttavien perinnöllisten riskitekijöiden esiintyvyyttä diabetesta sairastavilla henkilöillä.

Menetelmät. Tässä kirjassa esitetyt osatutkimukset tehtiin koko Suomea edustavassa FinnDiane (Finnish Diabetic Nephropathy Study Group) monikeskustutkimuksessa. Tutkimusaineisto koostuu aikuisista tyypin 1 diabetesta sairastavista henkilöistä sekä ei-diabeettisistä verrokkihenkilöistä, jotka edustavat suomalaista taustaväestöä. Tutkimukseen osallistuvilta kerättiin tietoa sekä sairaalan ulko- että sisäpuolelta hoidetuista bakteeri-infektioista käyttäen kahta eri rekisteriä: terveydenhuollon hoitoilmoitusrekisteristä (HILMO) sekä kansallisesta reseptilääkeostosrekisteristä (KELA). Tietoa diabeteskomplikaatioiden ilmaantuvuudesta, etenemisestä sekä riskitekijöistä kerättiin potilaskäyntien yhteydessä, mutta myös sairaala- ja avoterveydenhuollon potilasarkistoista. DNA-näytteet kerättiin veren valkosoluista ja bakteeriperäisten lipopolysakkaridien (LPS) aktiivisuus määritettiin ensimmäisen tutkimuskäynnin yhteydessä seeruminäytteestä.

Tulokset. Bakteeri-infektiot olivat tyypin 1 diabetesta sairastavilla henkilöillä noin kaksi kertaa yleisempiä ei-diabeettiseen taustaväestöön verrattuna. Infektioiden esiintyvyys kasvoi erityisesti potilailla, joilla oli diabeettinen munuaistauti ja/tai huono sokeritasapaino. Lisääntynyt antibioottien käyttö sekä kohonnut seerumin LPS-aktiivisuustaso olivat itsenäisiä riskitekijöitä sepelvaltimotaudille ja vaikealle diabeettiselle retinopatialle. Löysimme myös diabetesta sairastavia henkilöitä käsittävässä genomilaajuisessa assosiaatiotutkimuksessa (GWAS) potentiaalisen kytkennän kromosomilla 2 sijaitsevien geneettisten markkerien ja infektioherkkyyden välillä. Lisätutkimusten mukaan tämä infektioherkkyyteen vaikuttava kromosomikytkentä voisi liittyä IRAK1-signaalipolun aktiivisuuden säätelyyn.

Päätelmät. Bakteeri-infektioiden esiintyvyys on yleisempää tyypin 1 diabetesta sairastavilla henkilöillä taustaväestöön verrattuna. Toistuvat antibioottiostokset sekä korkea LPS-aktiivisuustaso liittyvät mikro- ja makrovaskulaaritautien kehittymiseen. Geneettiset tekijät kromosomilla 2 saattavat vaikuttaa diabetesta sairastavien henkilöiden infektioherkkyyteen.

ABSTRAKT

Bakgrund. Personer med diabetes har en högre risk att insjukna i bakterieinfektioner jämfört med grundbefolkningen. Hos personer utan diabetes har dessa infektioner associerats med mikro- samt makrovaskulära sjukdomar (t.ex. njursjukdom och hjärt- och kärlsjukdom) men infektionernas roll i uppkomsten av dessa sjukdomar är oklar. Denna roll kan ha en stor betydelse hos individer med diabetes som lider av både mikro- och makrovaskulära sjukdomar samt bakterieinfektioner mer frekvent än den övriga befolkningen. Prevalensen av bakterieinfektioner hos individer med specifikt typ 1 diabetes är dessutom oklar, likaså hur kronisk hyperglykemi påverkar prevalensen. Även genetiska faktorer som skulle kunna påverka bakterieinfektionsfrekvensen hos individer med diabetes är bristfälligt kartlagda.

Mål. Denna avhandlings syfte var att undersöka prevalensen av bakterieinfektioner hos individer med typ 1 diabetes, samt utreda hur dessa infektioner kunde påverka risken att insjukna i diabetisk njursjukdom, kranskärlssjukdom och diabetesretinopati. Vidare forskade vi huruvida vi kunde påvisa ett samband mellan vanliga punktmutationer i genomet och infektionskänslighet hos individer med diabetes.

Metoder. Studierna presenterade i denna avhandling gjordes inom den nationella multicenterstudien Finndiane (Finnish Diabetic Nephropathy Study Group). Forskningsmaterialet utgörs av vuxna individer med typ 1 diabetes samt kontrollindivider utan diabetes, som representerar den finska grundbefolkningen. För forskningen samlades information på bakterieinfektioner vårdade såväl inom som utanför sjukhus från två olika register: sjukhälsovårdens vårdanmälningsregister (HILMO) samt det nationella receptläkemedelsuppköpsregistret (uppehållet av folkpensionsanstalten [FPA]). Information gällande uppkomst och progression av diabeteskomplikationer samt deras riskfaktorer samlades i samband med kliniska studiebesök och från patientarkiv. DNA-prov togs från blodets leukocyter och aktiviteten på bakteriers lipopolysackarider (LPS) mättes från serumprov tagna i samband med deltagarnas första kliniska studiebesök.

Resultat. Bakterieinfektioner var ungefär två gånger vanligare hos individer med typ 1 diabetes jämfört med kontrollindivider utan diabetes. Infektionerna var mer frekventa hos individer med njursjukdom och/eller dålig sockerbalans. Frekventa antibiotikauppköp samt förhöjda nivåer av LPS-aktivitet var självständiga riskfaktorer för kranskärlssjukdom samt svår diabetesretinopati. I en genomomfattande associationsstudie (GWAS) på personer med diabetes hittade vi ett möjligt samband mellan varianter belägna på kromosom 2 och infektionskänslighet. Denna association mellan de genetiska loci vi fann samt infektionsfrekvens medierades potentiellt via signaleringsvägen *IRAK1*.

Slutsatser. Bakterieinfektioner är vanligare hos individer med typ 1 diabetes i jämförelse med grundbefolkningen. Frekventa antibiotikauppköp samt höga nivåer av LPS-aktivitet associerar starkt

med uppkomsten av mikro- samt makrovaskulära sjukdomar. Genetiska faktorer på kromosom 2 kan möjligtvis påverka infektionskänsligheten hos individer med diabetes.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Simonsen JR*, Harjutsalo V*, Järvinen A, Kirveskari J, Forsblom C, Groop PH, Lehto M. Bacterial infections in patients with type 1 diabetes: A 14-year follow-up study. BMJ Open Diabetes Res Care. 2015;3:e000067.

II Simonsen JR, Järvinen A, Harjutsalo V, Forsblom C, Groop P-, Lehto M. The association between bacterial infections and the risk of coronary heart disease in type 1 diabetes. J Intern Med. 2020;288:711-724.

III Simonsen JR, Järvinen A, Hietala K, Harjutsalo V, Forsblom C, Groop PH, Lehto M. Bacterial infections as novel risk factors of severe diabetic retinopathy in individuals with type 1 diabetes. Br J Ophthalmol. 2020;105:1104-1110.

IV Simonsen JR, Käräjämäki A, Antikainen A, Toppila I, Ahlqvist E, Prasad R, Mansour-Aly D, Harjutsalo V, Järvinen A, Tuomi T, Groop L, Forsblom C, Groop PH, Sandholm N, Lehto M. Genetic factors affect the susceptibility to bacterial infections in diabetes. Sci Rep. 2021;11:9464.

* Equal contribution

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Author's contribution

In study I the author was together with V. Harjutsalo responsible for data assembly, interpretation of the results and for writing the manuscript. In study II-III the author contributed to the assembly of the data, was the lead statistical analysist, interpreted the results and wrote the manuscript. In study IV the author was the lead statistical analysist, contributed to the design of the study, the validation and interpretation of the phenotypic data and results, and wrote the manuscript together with N. Sandholm and M. Lehto.

ABBREVIATIONS

AER	Albumin excretion rate
ATC	Anatomical chemical classification system
BMI	Body mass index
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CRP	C-reactive protein
DCCT	The Diabetes Control and Complications Trial Research Group
DNA	Deoxyribonucleic acid
DIREVA	The Diabetes Register Vaasa study
ECG	Electrocardiogram
eGFR	Estimated glomerular filtration rate
ETDRS	Early Treatment of Diabetic Retinopathy Study
FinnDiane	Finnish Diabetic Nephropathy Study Group
GFR	Glomerular filtration rate
GWAS	Genome-wide association study
HbA_{1c}	Glycosylated haemoglobin A_{1c}
HDL	High-density lipoprotein
HLA	Human leucocyte antigen
HR	Hazard ratio
ICD	International classification of diseases
IL	Interleukin
KDIGO	Kidney Disease: Improving Global Outcomes
LAL	Limulus amebocyte lysate
LDL	Low-density lipoprotein
LPS	(Bacterial) lipopolysaccharide
NF-κB	Nuclear factor-κB
NOMESCO	Nordic Medico-Statistical Committee classification system
RRs	Rate ratios
SGLT-2	Sodium-glucose cotransporter-2
SNP	Single nucleotide polymorphism
THL	National Institute for Health and Welfare
TLR	Toll-like receptor
WHO	World Health Organization

1. INTRODUCTION

Diabetes is one of the great pandemics of our age. Up to 8.3% of the global population were living with diabetes in 2014[1], and in 2015, the costs of the treatment of diabetes and diabetic complications equalled 1.8% of the global gross domestic product[2]. Diabetes is a broad term, covering several clinical phenotypes, each with their own clinical presentations, characteristics, and pathophysiology. Type 1 diabetes is the disease presenting usually in childhood, adolescence or early adulthood, due to external environmental factors initiating the autoimmune destruction of pancreatic beta cells in genetically susceptible individuals. This leads to the inability to produce sufficient amounts of insulin, resulting in chronic hyperglycaemia requiring external insulin treatment. The incidence of type 1 diabetes is increasing globally[3], and Finland has the highest incidence of type 1 diabetes in the world[4]. Type 1 diabetes has a massive impact on morbidity and mortality, which is mainly due to the chronic complications that develop and progress over the increasing duration of the disease[5] [6]. The chronic complications of diabetes are traditionally classified into microvascular complications (diabetic kidney disease, diabetic retinopathy, and diabetic neuropathy) and macrovascular complications (cardiovascular disease; stroke, coronary heart disease and peripheral artery disease). Although active research on the chronic complications of diabetes has been conducted up to decades already, and several risk factors for these complications have been ascertained, the pathogenesis behind these diseases are yet unclear and novel risk factors are still being discovered.

Bacterial infections have been shown to occur more frequently in individuals with diabetes, compared to the general population[7] [8] [9] [10] [11]. Although the mechanisms behind this susceptibility to infections are unknown, earlier studies have demonstrated that hyperglycaemia impairs the function of leukocytes, a paramount defending cell-line in the host defence against bacteria[12] [13] [14]. Bacterial infections, in turn, induce substantial inflammatory responses that result in the secretion of systemically circulating pro-inflammatory cytokines and proteins[15]. Inflammation has been shown to play an essential role in the pathogenesis of micro- and macrovascular disease in both individuals with diabetes as well as in non-diabetic individuals[16] [17] [18] [19] [20]. Of note, in the latter group, bacterial infections have been associated with both incident cardiovascular disease as well as acute kidney injury[21] [22]. In addition, membrane components of gram-negative bacteria, bacterial lipopolysaccharides, and their activity in serum have been associated with the progression and development of diabetic kidney disease as well as incident cardiovascular disease[23] [24] [25].

Although infections have been associated with cardiovascular disease and certain types of kidney disease in the general population, and inflammation has been hypothesized to play an important role in the development of micro- and macrovascular disease, the association between bacterial infections and chronic complications of diabetes is largely unknown. Furthermore, although it is commonly thought that infections are more common in individuals with diabetes, few studies have surveyed how this risk

applies to individuals with specifically type 1 diabetes, even though these individuals differ to other types of diabetes in several regards. Finally, the mechanisms behind the increased susceptibility to infections in diabetes is yet unclear.

The aim of the present doctoral thesis was to assess the incidence of bacterial infections in individuals with type 1 diabetes and to investigate the association of the infections with both micro- as well as macrovascular complications of diabetes. Finally, we explored whether common genetic factors associate with the susceptibility to bacterial infections in individuals with diabetes.

2. REVIEW OF THE LITTERATURE

2.1 Diabetes Mellitus

Diabetes mellitus is a broad term for a collection of metabolic diseases, characterised by hyperglycaemia[26]. Diabetes mellitus can be categorised into different classes depending on several clinical parameters, including the age of onset of diabetes, potential ketoacidosis at onset, the predominance of insulin resistance or insulin deficiency, and the presence of islet autoantibodies. Traditionally, diabetes has been divided into type 1 diabetes, type 2 diabetes and the less common forms of diabetes: Latent Autoimmune Diabetes of the Adult (LADA) and Maturity Onset Diabetes of the Young (MODY)[27]. Other forms, such as mitochondrial diabetes as well as secondary diabetes due to external factors (e.g., pancreatitis or glucocorticoid treatment) occur as well, although not as commonly as diabetes type 1 and 2. Type 1 diabetes usually presents in adolescence/early adulthood with considerable insulin deficiency, fast transition to dependence of external insulin therapy as well as a presence of islet autoantibodies. Type 2 diabetes on the other hand usually presents in adulthood with considerable increase in insulin resistance and is often associated with obesity. Although, notably, some individuals with type 2 diabetes exhibit a reduced insulin production instead and may develop diabetes in childhood, while some individuals with type 1 diabetes develop the disease in late adulthood. Due to the variance observed in the clinical presentation of the types of diabetes, there is a large overlap between the classifications of diabetes, and recent research has questioned these classifications using novel data-driven clustering methods[28].

Diabetes currently poses a tremendous challenge and concern for health care at a global level. Since 1980, the prevalence of diabetes has almost quadrupled (108 million to 422 million between 1980 and 2014)[29]. Alarmingly, this number has been predicted to continue to rise at a similar speed and the number of individuals with diabetes in 2040 has been estimated to exceed 640 million[30]. Most of the increase in the rising prevalence of diabetes is attributable to the global surge in the incidence of type 2 diabetes, although the prevalence of type 1 diabetes is increasing as well[31].

2.2 Type 1 diabetes

Overview, epidemiology and pathogenesis of type 1 diabetes

Diabetes Mellitus type 1 is one of the most common autoimmune disorders that affects roughly 1% of the general population and accounts for roughly 5-10% of all diabetes cases[32]. Previously called “childhood diabetes”, type 1 diabetes is characterised by its early onset, debuting usually in childhood or early adulthood, although it can present at any age. Contrary to other common autoimmune childhood diseases, type 1 diabetes has a male predominance[33]. During the past decades, the incidence of type 1 diabetes has been slowly increasing world-wide, and although in some countries this increasing trend

has begun levelling off, the incidence still increases annually by roughly 3.4%[31]. This also means a doubling of the incidence rate within 20 years.

In type 1 diabetes, environmental factors trigger an autoimmune assault on the pancreatic beta cells in genetically susceptible individuals[34]. It is noteworthy that although the disease is commonly considered an autoimmune disease, in roughly 10% of type 1 diabetes cases, no autoimmunity can be observed and are hence considered idiopathic[35]. Type 1 diabetes is a complex, polygenic disease where the inheritable factors have a generally low penetrance, due to which only 10-15% of individuals with type 1 diabetes have a first- or second-degree relative with type 1 diabetes. In monozygotic twins, the disease concordance has been estimated to be roughly 40%, with variation attributable to the age at onset of the disease[36]. Previous studies have identified over 50 genetic risk loci associating with the disease, of which the most significant locus is located within the HLA-complex (human leucocyte antigen) on chromosome 6, in which variants have been estimated to attribute to up to 40–50% of the genetic risk of the development of type 1 diabetes[37].

The destruction of the beta cells in individuals with a genetic predisposition is thought to be triggered by environmental factors[38], including viral infections and dietary factors such as vitamin D-deficiency [39 40]. Of note, vaccines have previously been thought to increase the risk for type 1 diabetes, however, extensive meta-analyses recently concluded that no association between childhood vaccines and the risk of type 1 diabetes could be seen[41]. An emerging hygiene hypothesis states that the improved hygiene during recent decades and consequently fewer infections in childhood, could predispose the individuals to autoimmune diseases, including type 1 diabetes[38]. Regardless of which trigger is involved, the resulting beta cell dysfunction leads to an insufficient secretion of insulin. Insulin, an anabolic peptide hormone produced from the cleavage of the C-peptide in the proinsulin molecule, regulates the glucose concentration in the blood by promoting the absorption of glucose into skeletal muscle, fat, and liver cells. The destruction of the beta cells, hence, impairs the transportation of glucose into specific tissue cells and causes glucose to accumulate in the blood, i.e., hyperglycaemia, which is the main clinical hall mark of diabetes and often persists over long periods of time, despite treatment with external insulin[42 43]. During persistent hyperglycaemia, glucose binds to the haemoglobin molecule in erythrocytes through a non-enzymatic glycation reaction, resulting in glycated haemoglobin (HbA_{1c}). This glycation product is widely used in clinical settings, as a marker for the evaluation of long-term glucose control. HbA_{1c} reflects the glucose control over the last 2-3 months, approximately, which is the average half-life of erythrocytes, and is reported as either a percentage or millimoles per mole (mmol/mol). In healthy non-diabetic individuals, HbA_{1c} is below 6.0%, however, in type 1 diabetes it's usually above 7%, and >10% in roughly a quarter of the individuals with type 1 diabetes[42]. The control of hyperglycaemia while avoiding hypoglycaemic events is one of the cornerstones and main goals in the clinical treatment of diabetes[44].

Finland has to this date the highest incidence of type 1 diabetes in the world with over 40 cases per 100,000 individuals being diagnosed annually[45]. However, this incidence has ceased to increase after 2005 in children under 15 years of age[46]. This finding has been postulated to potentially be due to changes in the environment and recommendation on vitamin D supplementation in Finland[47]. Interestingly, research has shown a remarkable increase of 33% in the incidence of type 1 diabetes in young Finnish adult individuals (age 18-39) from 1992 to 2007[48].

Clinical implications of type 1 diabetes

Type 1 diabetes has a tremendous impact on an individual's morbidity and mortality. Although novel treatment methods such as pancreatic transplantation are presently available for a few selected individuals, the diagnosis of type 1 diabetes is often accompanied with life-long insulin treatment and frequent physician visits, with associated blood and urine tests. The individuals continue to strive for optimal glycaemic control with careful glucose self-monitoring, dietary management as well as insulin titration, while balancing between the risk of hypoglycaemia and potentially severe neurological symptoms or hyperglycaemia and an increased risk for diabetic complications or acute ketoacidosis. It is no wonder that individuals with type 1 diabetes have a three-fold higher risk for mental health disorders, such as depression, compared to non-diabetic individuals[49]. This risk of poor mental health also seems to correlate with poor glycaemic control[50]. At present, type 1 diabetes is still associated with a high mortality[51], which is mainly attributable to the development and progression of the chronic complications of diabetes.

2.3 Chronic complications of diabetes

Long-lasting diabetes and chronic hyperglycaemia inflict extensive damage on different cells and tissues over time. Together, with other risk factors, both environmental and genetic, they give rise to the development of the chronic complications of diabetes[52]. The complications are traditionally classified into macrovascular complications (cardiovascular disease: cerebrovascular disease, coronary heart disease, and peripheral artery disease) and microvascular complications (diabetic retinopathy, diabetic kidney disease, and diabetic neuropathy). Although these diseases affect different organs, they all have been found to tightly associate to one another and share similar risk factors, albeit to different degrees: age, long duration of diabetes, poor glycaemic control, obesity, dyslipidaemia, hypertension and smoking[53 54 55 56]. Additionally, inflammation has been associated with the development of both micro- and macrovascular diabetic complications[16 17 19 57]. Genetic factors also play a major role in the development of different complications, although which genetic loci and to which degree they affect the risk of the development of the complication varies greatly between the complications.

A fundamental aspect of the treatment of diabetes is the prevention of the development and progression of diabetic complications[58]. A landmark study published in 1993 by The Diabetes Control and Complications Trial Research Group (DCCT) demonstrated, in a large prospective setting, the

importance and impact of optimal glycaemic control on the risk of developing late diabetic complications[59]. The treatment of other dynamic risk factors is as important as the optimisation of glycaemic control, and intensive treatment of hypertension, obesity and dyslipidaemia as well as cessation of smoking are highly recommended in Finland[60]. The treatment goals also intensify as diabetic complications emerge and progress.

2.3.1 Diabetic kidney disease

The function of the kidney

The kidney is responsible for numerous vital processes that are necessary for the maintenance of homeostasis. These processes include filtration of waste from the blood, reabsorption of ions, glucose, and nutrients from the urine, regulation of blood pressure and acid-base homeostasis, upholding the balance of electrolytes and fluids, stimulation of erythropoiesis through the production and secretion of erythropoietin, and finally, the generation of the biologically active vitamin D metabolite. Filtration of blood takes place in glomeruli, a comprehensive network of capillaries within the nephrons of the kidney. In the glomerulus, blood is filtered through the glomerular filtration barrier into the Bowman's capsule, from which the filtration product, called the primary urine, is passed on to the proximal tubule. The glomerular filtration barrier is a complex structure consisting of three layers: the endothelial cells containing small openings (fenestrae) that freely permit the passage of small molecules, electrolytes and water; the glomerular basement membrane, a matrix of proteins separating the vascular space from the urinary space; and finally, the foot processes of the podocytes forming slit diaphragms that play an important part in the filtration barrier function on the urinary side of the glomerular filtration barrier.

Overview and pathophysiology of diabetic kidney disease

Diabetic kidney disease is a common chronic complication of diabetes, affecting up to a third of all individuals with type 1 diabetes[61]. Many consider diabetic kidney disease to be the most devastating complication, as it, in addition to being the most common cause of end-stage renal disease (ESRD) world-wide, also greatly increases the risk for both all-cause mortality as well as cardiovascular disease[62 63]. Furthermore, diabetic kidney disease gives rise to several kidney-function related secondary complications, which increases morbidity and lowers the quality of life, including anaemia, secondary hyperparathyroidism, fluid retention and swelling, hyperkalaemia and hypertension (**Fig 1**)[61]. The development of diabetic kidney disease takes time, often decades, and the prevalence increases with age. In individuals with longer durations of type 1 diabetes, the disease already affects the vast majority, and after 50 years of type 1 diabetes duration, 70% of the individuals suffer from diabetic kidney disease[64]. Fortunately, in individuals with type 1 diabetes, the incidence rates of diabetic kidney disease have had a decreasing trend over the last decades, most likely due to improved management of hyperglycaemia and hypertension as well as earlier detection of diabetic kidney disease[65].

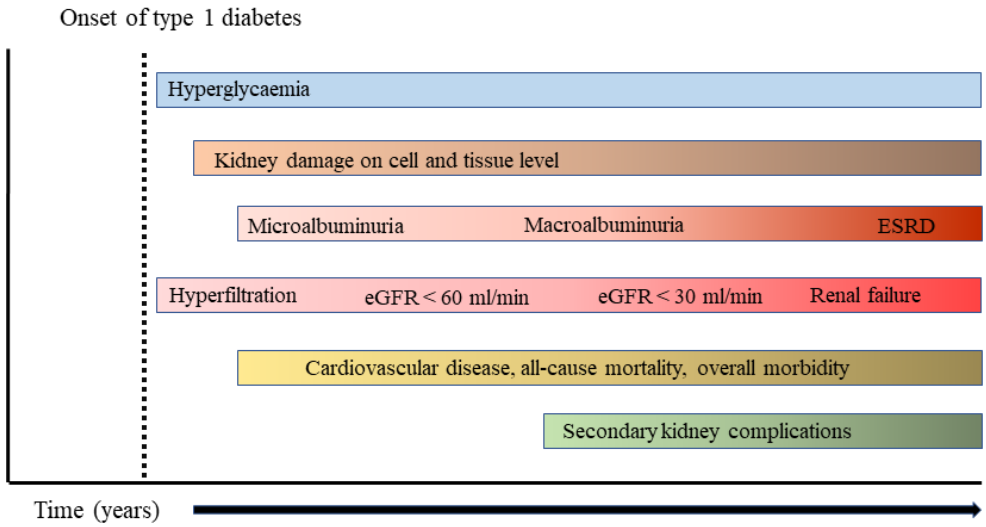


Figure 1. *Schematic figure of the emergence of the clinical aspects attributable to diabetic kidney disease and related diseases. Adapted from Alicis et al*[61]*. ESRD indicates End-stage Renal Disease; and eGFR, Estimated Glomerular Filtration Rate. Secondary kidney complications include hypertension, anaemia, secondary hyperparathyroidism, hyperkalaemia, fluid retention and oedema.*

Clinically, diabetic kidney disease leads to loss of protein (albumin) in the urine (albuminuria) and a progressive loss of kidney function[66]. In diabetic kidney disease, the kidneys' glomerular filtration barriers are damaged due to the diabetic milieu as well as other metabolic and environmental assaults. These factors result in the morphological hallmarks observed in diabetic kidney disease: thickening of the glomerular basement membrane, loss of podocyte foot processes, mesangial cell expansion and associated excessive formation of extracellular matrix, and finally, glomerulosclerosis, the scarring of the glomeruli[67]. In addition to these, tubulointerstitial fibrosis and tubular atrophy are often also observed. Previously, many considered the glomerular filtration rate and the urinary albumin excretion rate (AER) to reflect different aspects of the diabetic kidney disease pathology: while the reduced glomerular filtration rate was thought to stem from glomerulosclerosis, albuminuria was considered to be caused mainly due to metabolic injury to the podocytes, foot process effacement, and the consequential loss of slit diaphragms permitting the translocation of albumin into the primary urine[68]. Evidence, however, also suggest that all cell types present in the Bowman's capsule – and their dysfunction, could participate in the development of glomerulosclerosis and the resulting decline in kidney function[69]. The exact pathophysiologic mechanisms behind albuminuria and the decline in kidney function are yet unclear.

Interestingly, in roughly 40% of individuals with type 1 diabetes, renal hyperfiltration, an abnormally high filtration rate, is detectable in the early stages of the disease, and has been thought to reflect increased intraglomerular pressure and intrarenal hypertension[70]. Hyperfiltration has been considered a significant risk factor for the development of diabetic kidney disease[71], and is thought to stem from decreased tubuloglomerular feedback, involving the macula densa and the sodium-glucose cotransporter-2 (SGLT-2) in the proximal tubule[70]. The SGLT-2 protein reabsorbs approximately 90% of the glucose from the urine, together with sodium. In diabetes, glucose is abundant in the urine, and the increased reabsorption of the glucose also leads to an increased sodium reabsorption in the proximal tubule. As a consequence, sodium delivery to the macula densa is decreased, which causes the macula densa to strive to increase the glomerular perfusion by causing vasodilation of the afferent arteriole and subsequently, increasing the glomerular filtration rate as well as the energy expenditure. Simultaneously, the synthesis and secretion of renin is increased, subsequently causing vasoconstriction through the effect of angiotensin II, resulting in increased intraglomerular pressure as well as filtration rate.

Classification of diabetic kidney disease

With the progression of diabetic kidney disease over time the level of albuminuria increases while the kidney function decreases. Certain levels and thresholds of AER were previously used when categorising the severity of diabetic kidney disease into normal urinary AER, microalbuminuria, macroalbuminuria and finally, ESRD, which is defined as the time when the need for kidney replacement therapy emerges, i.e., either dialysis treatment or a kidney transplant is required (**Table 1A-I**). This categorization of kidney disease is important to distinguish from the categorization of other types of chronic kidney disease due to other disease, where the classification is performed according to kidney function (**Table 1A-II**). Kidney function is typically measured using glomerular filtration rate (GFR), defined as the fluid volume filtered through the glomerulus into the Bowman's capsule per unit time. This can be invasively measured e.g., using inulin infusion- and urinary clearance measurements, although, more commonly, an estimation of the filtration rate in the glomeruli (estimated glomerular filtration rate [eGFR]) is measured based on the serum creatinine or cystatin-C level, as it only requires an easily obtainable serum sample as opposed to the more arduous inulin clearance measurement or creatinine clearance measurement from a 24-hour urine collection. Based on the eGFR, expressed as ml/min/1.73 m^2, kidney function was categorized as normal (≥90), mildly reduced (60-89), moderately reduced (30-59), severely reduced (15-29) and finally, as renal failure (<15 ml/min/1.73 m^2).

It is, however, noteworthy, that even though renal function usually declines together with an increasing AER, some individuals exhibit a remarkably preserved kidney function regardless of the level of AER, while some individuals develop severe chronic kidney disease without significantly elevated AER[61]. The different resulting phenotypes of kidney disease also have different prognosis and risk of kidney

disease progression. The international organization Kidney Disease: Improving Global Outcomes (KDIGO) addressed this issue in their revised guidelines for the classification of chronic kidney disease, taking both albuminuria as well as kidney function into account when estimating the prognosis of chronic kidney disease (**Table 1B**)[72]. The different categories of chronic kidney disease, including diabetic kidney disease, were also revised. The levels of albuminuria were renamed into A1, normal to mildly increased (previously normal AER); A2, moderately increased (previously microalbuminuria); and A3, severely increased (previously macroalbuminuria). Corresponding reclassifications were made to classifications based on eGFR-categories. The updated classification system takes both albuminuria and kidney function into account when predicting the prognosis of diabetic kidney disease. Furthermore, the organization standardized the nomenclature and terminology referring to kidney disease: the use of the word "kidney" was recommended, when referring to kidney diseases, instead of the previously used "renal" or "nephron". Furthermore, "kidney failure" was preferred over "end-stage renal disease". However, at the time of the conduction of studies I-IV, the classification of diabetic kidney disease according to the level of albuminuria was used and therefore, this classification will also be used in this thesis.

Table 1.

A) Classification of the stage of I) DKD and II) CKD according to AER and GFR, respectively.

I.	Stage of DKD	AER
	Normal AER	<20 µg/min or <30 mg/24 h
	Microalbuminuria	≥20 µg/min or ≥30 mg/24 h
	Macroalbuminuria	≥200 µg/min or ≥300 mg/24 h
	ESRD	Onset of kidney replacement therapy (Dialysis or renal transplant)
II.	Stage of CKD	GFR (ml/min/1.73 m^2)
	Stage 1 - Normal	≥90
	Stage 2 - Mildly Reduced	60-89
	Stage 3 - Moderately reduced	30-59
	Stage 4 - Severely Reduced	15-29
	Stage 5 - Renal failure	<15

DKD indicates diabetic kidney disease; AER, albumin excretion rate; ESRD, end-stage renal disease; CKD, chronic kidney disease; GFR, glomerular filtration rate.

B) The prognosis of chronic kidney disease, by the levels of albuminuria and kidney function, and the stages of chronic kidney disease, according to the Kidney Disease: Improving Global Outcomes (KDIGO) organization[72].

Classification and categorisation of chronic kidney disease according to eGFR and albuminuria			Persistent albuminuria, expressed as mg/24 h or the urinary albumin-to-creatinine ratio (mg/g)		
			A1	A2	A3
			Normal to mildly increased	Moderately increased	Severely increased
eGFR categories, expressed as ml/min/1.73 m^2			<30 mg/24 h or <30 mg/g	30-300 mg/24 h or 30-300 mg/g	>30-300 mg/24 h or >30-300 mg/g
G1	Normal or high	≥ 90			
G2	Mildly decreased	60-89			
G3a	Mildly to moderately decreased	45-59			
G3b	Moderately to severely decreased	30-44			
G4	Severely decreased	15-29			
G5	Kidney failure	< 15			

eGFR indicates estimated glomerular filtration rate. Green colour indicates low risk, yellow indicates moderate risk, orange indicates high risk and red indicates very high risk of chronic kidney disease progression.

Risk factors for diabetic kidney disease

As in other diabetic complications, long duration of diabetes, poor glycaemic control, hypertension, and high age are fundamental risk factors for the development and progression of diabetic kidney disease[73]. Smoking and dyslipidaemia are also considered significant risk factors for the onset of the disease[74 75]. Another important risk factor for the development of not only diabetic kidney disease but chronic kidney disease over-all, is prior acute kidney injury, which is characterized by a sharp and sudden reduction in renal function, clinically defined as a substantial increase in serum creatinine or and most often, a parallel decrease in urine excretion. Behind the causes for acute kidney injury lie a myriad of aetiological possibilities, such as hypovolemia, infections or renal ischaemia due to septic shock or cardiac insufficiency. Acute kidney injury and chronic kidney disease can, to some extent, be considered

as a continuum and are clinically overlapping as chronic kidney disease is an important risk factor for acute kidney injury in critically ill patients (acute-on-chronic kidney injury), and vice versa: chronic kidney disease can be caused by acute kidney injury[76 77].

Genetics seem to play a substantial role in the pathophysiology of diabetic kidney disease. Repeated studies have found diabetic kidney disease to aggregate in families across different ethnic backgrounds, strongly advocating for the involvement of genetic factors in the development of the disease[78]. Using both candidate gene studies as well as more modern genome-wide association studies (GWAS), over 150 genes have been demonstrated to associate with diabetic kidney disease[78]. It is also noteworthy that previous research has found that up to 40% of AER variability can be explained by common genetic variations (single nucleotide polymorphisms, SNPs)[79].

Inflammation in diabetic kidney disease

Although diabetic kidney disease is not considered an inflammatory disease, inflammation appears to play an essential role in the disease's pathophysiology, driven first and foremost by the innate immunity[80 81]. The milieu in the diabetic kidney has been demonstrated to increase the secretion and release of cytokines and chemokines attracting monocytes, macrophages and lymphocytes to the kidney. These cells, particularly the macrophages, are activated in the diabetic kidney by the pro-inflammatory conditions caused by hyperglycaemia and associated renal cell injury. Once activated, the immune cells secrete proinflammatory cytokines and reactive oxygen species, initiating a cascade leading to kidney cell injury as well as chronic and unresolved renal fibrosis, finally resulting in glomerulosclerosis and podocyte injury[80 81]. Of note, the accumulation of macrophages in kidney tissue has been seen to associate with the increasing severity of glomerulosclerosis, and the magnitude of this accumulation correlates with both proteinuria as well as the decline of glomerular filtration rate[82 83].

Clinical treatment strategies of diabetic kidney disease

For all individuals with type 1 diabetes in Finland, comprehensive follow-up visits for the clinical management of their diabetes with at most 1-year intervals are recommended, and if necessary, more frequently[60]. At the visits, clinical signs of the development of chronic diabetic complications, including diabetic kidney disease, are routinely screened for. Blood samples as well as spot urine collections are used to determine the eGFR and AER, respectively, allowing the detection of diabetic kidney disease. To avoid the effect of confounding factors and other causes of albuminuria (e.g., menstruation or urinary tract infection), albuminuria should be detectable in two out of three urine collections before diagnosis of micro- or macroalbuminuria. Although kidney biopsy is the gold standard for diagnosing diabetic kidney disease, the diagnosis is also routinely made based on careful clinical and laboratory evaluation. At the detection of micro- or macroalbuminuria, therapeutic strategies include the effective treatment of risk factors: start of blood pressure lowering nephroprotective medication (angiotensin-converting-enzyme inhibitor or angiotensin receptor blocker), medication lowering low-density lipoprotein (LDL)

concentrations (statins), treatment and monitoring of blood pressure as well as dietary recommendations for optimal glycaemic control, and avoidance of excess protein intake depending on the stage of diabetic kidney disease[61 84]. When the disease advances to renal failure, the individual requires either dialysis treatment or a kidney transplant for the removal of waste from the blood and survival. It is noteworthy that microalbuminuria is still a reversible condition and regression to a previous lower level of albuminuria has been found to have beneficial effects on cardiovascular disease morbidity and mortality[85].

2.3.2 Cardiovascular disease

Overview and pathophysiology of cardiovascular disease and atherosclerosis

Cardiovascular disease is the greatest cause of mortality and morbidity in the Western world[86]. Cardiovascular disease is a collective term for diseases affecting the heart, brain, and peripheral arteries: coronary heart disease and other cardiopathies, cerebrovascular diseases, and peripheral artery disease, respectively. Of these diseases, coronary heart disease and cerebrovascular diseases are the most common, constituting up to 75-80% of cardiovascular disease[86]. Atherosclerosis, the single largest aetiology behind coronary heart disease and cerebrovascular diseases, as well as cardiovascular disease over-all, represents approximately 80% of cardiovascular disease world-wide. Although atherosclerosis is the main aetiology of cardiovascular diseases, other diseases do contribute to the global cardiovascular disease burden, including atrial fibrillation and the subsequent cardioembolic strokes and thrombosis, atypical cardiomyopathies as well as other causes of heart failure.

Atherosclerosis is a progressive disease, where chronic atheroma accumulation and damage to the intima in arteries lead to the stenosis of the blood vessel, impairing the circulation in the affected area or organ, e.g., the coronary arteries in coronary heart disease. The precise mechanisms through which atherosclerosis develops is still undetermined, however, a common scientific consensus, at present, is that collection of cholesterol-rich apolipoprotein B-particles within the intima in arteries is one of the main mechanistic events in the pathogenesis[87]. This is followed by leukocytes invading the intima, instigating an inflammatory process[19]. Of the monocytes invading the intima, some differentiate into macrophages that ingest lipids, thus becoming ‘foam cells’; while others secrete metalloproteases that degrade components of the extracellular matrix. Invading CD4 T-cells proliferate and secrete cytokines, which leads to smooth muscle cells migrating into the intima, proliferating and generating fibrous products, thus resulting in the thickening of the vascular wall and narrowing of the lumen. The death of leukocytes and muscle cells generates necrotic cores of cell debris, which continues and upholds the inflammatory process. This produces so called fatty streaks, which progress into plaques. These plaques cause progressive obstruction of the arterial lumen and may also rupture or erode and induce thromboembolic complications, i.e., arterial occlusion with consequent ischaemia, of which the most

feared events are coronary ischaemia (acute coronary syndrome) and cerebral stroke. The main organs affected by atherosclerosis include the heart, kidney, brain and periphery in the lower limbs.

Diabetes and cardiovascular disease

Diabetes has long been strongly associated with an increased risk of cardiovascular disease[88 89]. Individuals with diabetes have a two-times higher risk of dying from cardiovascular disease compared to non-diabetic controls (NDCs),[90] and previous studies have estimated that cardiovascular disease explains up to 60% of lost life years in diabetic individuals[91]. Of note, the pathogenesis of cardiovascular disease has been found to be slightly altered in diabetes, and there are differences in the development of cardiovascular disease between diabetic individuals and non-diabetic individuals[92]. One of the main differences between individuals with and without diabetes, in regard to risk factors for cardiovascular disease, is the presence of chronic hyperglycaemia. Although hyperglycaemia has been disputed and, over the years, regarded as a controversial risk factor for cardiovascular disease[93], at present, considerable scientific evidence supports hyperglycaemia as a substantial risk factor for cardiovascular disease, especially in type 1 diabetes[94]. In relation to this, cardiac autoimmunity, defined in this context as the presence of cardiac autoantibodies, has been shown to correlate with poor glycaemic control and to increase the risk for cardiovascular disease[95]. Interestingly, diabetes has also been found to cause chronic heart failure independently of coronary heart disease, aptly called "diabetic cardiomyopathy", mainly attributed to chronic metabolic insult resulting in structural changes leading to abnormal systolic as well as diastolic function, and subsequent heart failure[96]. Individuals with type 1 diabetes have also been found to exhibit subclinical cardiovascular disease findings, more frequently, compared to the general population, including coronary artery calcification, increased carotid intima-media thickness, and endothelial dysfunction, which are considered to be signs of early cardiovascular disease[97].

Cardiovascular autonomic neuropathy, a presentation of diabetic neuropathy in the nerves participating in the regulation of cardiovascular functions, is another unique feature in diabetic cardiovascular disease. The prevalence has been estimated to be at least 20% in individuals with type 1 and type 2 diabetes, however, with increasing diabetes duration and age, the prevalence may be as high as 65%, making it a noteworthy risk factor for cardiovascular disease in diabetes[98]. Cardiovascular autonomic neuropathy's clinical manifestations range from mild orthostatic hypotension and resting tachycardia, to prolongation of the QT-interval, silent cardiac ischaemia, and sudden death. The presence of cardiovascular autonomic neuropathy dramatically increases the risk of cardiovascular mortality five-fold for the affected individuals[99 100].

A final difference regarding the presentation of coronary heart disease in individuals with diabetes versus non-diabetic individuals is the anatomy of the atherosclerotic plaques. Individuals with diabetes have a higher incidence of multivessel disease, greater numbers of peripheral lesions as well as a greater atheroma burden[101]. Due to these characteristics, as well as the presence of other diabetes-specific risk

factors such as hyperglycaemia and cardiovascular autonomic neuropathy, coronary heart disease is considered more lethal in diabetes.

In addition to the differences in the development of cardiovascular disease between diabetic and non-diabetic individuals, the pathogenesis of cardiovascular disease is also considered to be different to some extent between type 1 and type 2 diabetes, as these forms of diabetes have considerable differences in clinical presentation and phenotype[97]. In fact, cardiovascular disease has been found to be disproportionately more frequent in type 2 diabetes compared to type 1 diabetes, likely due to the prevalence of obesity and dyslipidaemia associable to type 2 diabetes, while in individuals with type 1 diabetes a substantial part of the risk of cardiovascular disease in type 1 diabetes has been considered attributable to the presence of diabetic kidney disease[62]. Of note, non-diabetic chronic kidney disease has also been demonstrated to function as an independent risk factor for cardiovascular disease in the general population[102].

Cardiovascular disease and inflammation

The role of inflammation in the pathophysiologic events of cardiovascular disease and atherosclerosis has been strongly discussed both for and against, especially during recent years. Numerous previous publications have advocated that inflammation, acute and chronic, is a key element in the pathophysiology of atherosclerosis[103 104 105]. Leukocytes have been found to participate in all stages of the development of atherosclerosis[106]. In addition to the local inflammatory processes instigated by leukocytes, several studies have demonstrated a strong association between atherosclerosis and inflammation markers, such as C-reactive protein (CRP) and interleukin 6 (IL-6). CRP has been found to function as a predictor of incident cardiovascular disease[107], and individuals with high levels of CRP have a four-fold higher risk of coronary heart disease compared to individuals with low CRP-concentrations[108 109]. Comprehensive meta-analyses have demonstrated IL-6, in turn, to be strongly associated to coronary heart disease[110]. A strong supporter of the inflammation-cardiovascular disease hypothesis was a large-scale Mendelian randomization study concluding that higher circulating concentrations of IL-6 receptors had a significant protective role against coronary heart disease, and demonstrated that IL-6 signalling pathways play a causal role in the pathogenesis of coronary heart disease[111]. This effect was thought to stem from the increased concentration of IL-6 receptors causing less IL-6 signalling, and hence, lower circulating CRP.

Inflammation is also tightly connected to other cardiovascular disease risk factors. Dyslipidaemia has been frequently associated with low-grade inflammation and angiotensin II, a key vasoconstrictor and mediator of hypertension that has been found to cause intimal inflammation, potentially linking hypertension and cardiovascular disease also through inflammatory pathways[112]. Further of interest is that cardiovascular autonomic neuropathy has been postulated to have strong associations to

inflammation as well[113], potentially underlining the role of inflammation in diabetic coronary heart disease.

Some research, however, has implicated that inflammation would, at best, only be a bystander in the pathogenesis of atherosclerosis. Recent Mendelian randomization studies found that levels of CRP seem to play little to no causative role in the development of cardiovascular disease[114 115]. Regarding these studies using inflammation markers as measurements of levels of inflammation (including the Mendelian randomization study demonstrating IL-6 as a causal factor for coronary heart disease[111]), it is necessary to exercise caution in the interpretation of results and drawing conclusions on causality. The markers differ greatly in their position in the inflammatory pathways and cascades. Although all markers, to some, degree reflect systemic inflammatory activity, several markers, including CRP, are nonspecific products far downstream in the signalling cascade and only reflect a part of the inflammatory burden. The levels of the measurements of these markers entail no information on the underlying pathways or inflammation sites causing the upregulation of the secretion of the markers[116]. Compared to CRP, IL-6 is considered an upstream marker that stimulates downstream inflammation pathways and cascades and can, therefore, be considered a more reliable estimate of systemic inflammation. However, even taking its upstream position into account, IL-6 suffers the same limitation as other inflammatory markers in that it is not specific for certain sites or anatomical locations.

Clinical management of cardiovascular disease in diabetes

Even though diabetes is a substantial risk factor for cardiovascular disease, it's detection and diagnosis in individuals with diabetes can be challenging as the disease may present with atypical or even silent symptoms during both myocardial as well as cerebral ischaemia[117 118]. Coronary heart disease is usually suspected based on angina pectoris symptoms experienced during physical exertion, however, these symptoms usually only occur after the obstruction of the lumen of the coronary artery exceeds 70%[119]. Individuals with diabetes have more diffuse and peripheral coronary artery atherosclerosis, compared to non-diabetic individuals, and therefore they display clinical symptoms less frequently or not at all. Due to this, as well as to the high risk of coronary heart disease, electrocardiograms (ECGs) are recommended for all adult individuals with diabetes with 1 to 3-year intervals[60]. The diagnosing methodology of cardiovascular disease depends on the affected site (heart, brain, peripheral artery), however, in the case of a severe disease requiring invasive treatment, each of these sites are typically investigated with angiography and/or modern radiologic imaging techniques (computer tomography or magnetic resonance imaging). After the diagnosis of an atherosclerotic cardiovascular disease, individuals are assigned preventive antithrombotic therapy as well as effective treatment of all potential risk factors. Surgical interventions include percutaneous artery intervention and intra-artery stenting, artery bypass graft-surgery or endarterectomy.

2.3.3 Diabetic retinopathy

Overview and pathophysiology of diabetic retinopathy

Diabetic retinopathy is arguably the most common chronic diabetic complication, affecting up to 86% of individuals with type 1 diabetes[120]. Diabetic retinopathy arises through various vascular abnormalities in the retina and is traditionally classified into non-proliferative diabetic retinopathy as well as the more progressed stage, proliferative diabetic retinopathy.

In non-proliferative diabetic retinopathy, microvascular injuries and abnormalities arise in the retina, including local haemorrhages, hard lipid exudates as well as microaneurysms. Microaneurysms and haemorrhages further lead to blood and fluids leaking into the surrounding retinal tissue. Macular oedema is caused when fluid leaks into the macula, the area in the retina containing the fovea, which is necessary for acute vision. This consequently causes lowered vision in the affected individuals and can occur across all stages of diabetic retinopathy[121]. Macular oedema is one of the most common causes of vision loss in working age individuals, and together with proliferative diabetic retinopathy, the condition is classified as severe diabetic retinopathy. Proliferative diabetic retinopathy, an advanced form of non-proliferative retinopathy, is caused by angiogenesis, proliferating blood vessels, in the retina. This neovascularisation is stimulated by the vascular endothelial growth factor signalling protein (VEGF), which is upregulated in diabetes mainly due to local hypoxia or ischaemia[122], in turn caused by hyperglycaemia. VEGF is secreted by many cells in the retina and promotes both angiogenesis as well as an increase in the permeability of the blood vessels. The newly formed blood vessels are fragile and may easily bleed into the vitreous body, thereby hindering the light from reaching the retina, and therefore causing vision loss. Distinguishing non-proliferative retinopathy from proliferative retinopathy has substantial clinical implications as the treatment of the latter differs greatly from the former.

Inflammation in diabetic retinopathy

As in other chronic diabetic complications, inflammation and inflammatory-like processes have been postulated to play a central role in the pathogenesis of diabetic retinopathy, and during recent years, scientific evidence demonstrating the role of inflammation in the development of diabetic retinopathy has been accumulating[123] [124] [125]. Inflammatory changes in the diabetic retina may precede the microvascular lesions detected at a later stage in the disease. Within one week after the onset of diabetes in mice, neutrophils adhere to the endothelial cells in the blood vessels in the retina, initializing a cascade, ultimately resulting in worsened capillary perfusion as well as the breakdown of the blood-retinal barrier, which contributes to macular oedema[126] [127]. This leukocyte adhesion is a key process in the pathogenesis of diabetic retinopathy, and inhibition of the adhesion has been shown to prevent the development of hallmark features of diabetic retinopathy: retinal endothelial cell injury and death[128]. Moreover, experimental models have demonstrated that endothelial cells in the retina activate the

nuclear factor-κB (NF-κB) pathway in response to elevated glucose concentrations, increasing local inflammatory processes[125]. Levels of tumour necrosis factor alpha (TNF-alpha) in the retinal tissue of diabetic rats are much higher compared to non-diabetic rats and systemic treatment with nonsteroidal anti-inflammatory drugs in diabetic rats reduces leukocyte adhesion as well as blood-retinal barrier breakdown[129]. Furthermore, the ocular tissue in individuals with diabetic retinopathy has been demonstrated to have much higher levels of inflammatory markers, compared to non-diabetic individuals; and the concentration has been shown to correlate with the severity of diabetic retinopathy[130].

Clinical management of diabetic retinopathy

Screening and diagnosing of diabetic retinopathy are based on ophtalmoscopy and/or fundus photographs, images of the retina, where vascular abnormalities relating to both proliferative as well as non-proliferative retinopathy can be detected. The ETDRS-scale (Early Treatment of Diabetic Retinopathy Study) is a common scale used to classify the severity of diabetic retinopathy based on the findings in these fundus photographs (**Table 2**). The cornerstones of the treatment of diabetic retinopathy are mainly the preventive and effective treatment of the risk factors. Severe diabetic retinopathy can be treated with both anti-VEGF oral medications as well as photocoagulation therapy.

Table 2. A summary of the levels of the ETDRS-scale (Early Treatment of Diabetic Retinopathy Study) and clinically detectable findings in fundus photos of each level. Adapted from Davis et al.[131].

Level	Clinical severity (ETDRS-scale)	Detectable signs of diabetic retinopathy
10-20	No retinopathy	No clinical signs of retinopathy
20-35	Very mild-mild non-proliferative retinopathy	Microaneurysms, hard exudates and/or mild retinal haemorrhages
35-47	Moderate non-proliferative retinopathy	Moderate retinal haemorrhages, mild intraretinal microvascular abnormalities
53	Very severe non-proliferative retinopathy	Severe retinal haemorrhages, moderate to severe intraretinal microvascular abnormalities
61-85	Proliferative retinopathy	Newly formed blood vessels, initially only locally in small areas but with increasing severity of retinopathy larger areas are covered.

2.3.4 Diabetic neuropathy

Overview of diabetic retinopathy

Diabetic neuropathy refers to the chronic damage to mainly peripheral and autonomic nerves caused by the diabetic milieu, resulting in the dysfunction of the innervated tissues, limbs and organs. The prevalence of diabetic neuropathy has been estimated to be between 30-50% in individuals with diabetes[132]. These numbers, however, are somewhat misleading as they address all forms of diabetes and diabetic neuropathy is considered to be more common in type 1 diabetes compared to type 2 diabetes[133]. Some studies even suggest that closer to 100% of individuals with type 1 diabetes will develop diabetic neuropathy during their lifetime, also making diabetic neuropathy, arguably, more common than diabetic retinopathy and, thus, the most common chronic diabetic complication[134].

The symptoms of diabetic neuropathy depend on which nerves are affected. Most commonly, diabetic neuropathy presents as symmetric and mainly sensory peripheral polyneuropathy. In practice, this means reduced sensation in the affected individual's hands and feet, and/or chronic neuropathic pain. Autonomic neuropathy is also common, although due to the heterogenous clinical presentation, it may be difficult to detect as depending on which nerve is affected, the individuals may experience a range of symptoms relating to gastrointestinal dysfunction (gastroparesis, constipation, diarrhea, incontinence), genitourinary dysfunction (bladder atony, impotence), sudomotor dysfunction or cardiovascular autonomic neuropathy[135].

2.4 The human immune system, bacterial infections and inflammation

Overview of the components of the human immune system

A detailed review of the functions and pathways of the human immune system and its interactions with microbial pathogens is beyond the scope of the present thesis, although a brief summary is needed. The human body contains abundant bacterial growth in its cavities, mainly in the gastrointestinal system, but also on the skin. Normally, these bacteria do not give cause to an inflammatory response, and in fact are in several ways vital for the maintenance of homeostasis as well as production of essential short-chain fatty acids and vitamins, such as vitamin K, in the large intestine. However, when bacteria enter anatomic locations that are normally sterile and encounter white blood cells, an inflammatory response is triggered. This response is first initiated by the first line of defence, the innate immune system, which is followed by the adaptive immune system response. The innate immune system is a collective term for several different components, including antibodies, chemokines and cytokines, immunologic cells, and finally, the complement system. Leukocytes participating in the innate defence against microbial pathogens, in turn, include: I) antigen presenting cells, responsible for presenting bacterial components on the surface and further activating other immune cells; II) natural killer cells, that directly neutralize pathogens, and finally; III) monocytes, macrophages and neutrophils responsible for phagocytosis, i.e.,

the process of ingesting and eliminating particles, substances and microbes. In addition to immunologic cells, epithelial cells play an important role in the innate immune system, as they can secrete cytokines that modulate and activate immunologic reactions[136]. The adaptive immune system in turn is activated mainly by the innate immune system. The adaptive system consists of subtypes of lymphocytes: B-cells and T-cells[137]. B-cells secrete immunoglobulins, such as immunoglobulin G and M (IgG and IgM, respectively), that inactivate and opsonize pathogens, allowing more effective phagocytosis. T-cells in turn initiate a cell-mediated response and depending on the target can either kill pathogens directly or activate other leukocytes, e.g., monocytes. Monocytes, once activated, can differentiate into macrophages, which are active phagocytes that also further stimulate and potentiate the innate immune response by activating other leukocytes (natural killer cells and dendritic cells) and the complement system, which enables the opsonization of pathogen particles. Macrophages also secrete soluble pattern recognition molecules that augment phagocytosis overall, as well as produce cytokines and proteins that modulate the immunologic response towards the pathogen and directly neutralise the potential invader. The transformation into macrophages also increases the phagocytotic capabilities of the cell. An important cell line in the defence against invasion of bacterial pathogens are the neutrophils of the innate immune system. Neutrophils constitute up to 50-70% of all leukocytes in the body, are highly mobile and present in many anatomical sites, including the systemic circulation. They are typically the first immunologic cells recruited to the infection or inflammation site, establishing the first line of defence. Neutrophils, like the macrophages, are important phagocytes and secrete cytokines that regulate the inflammatory response. Their importance in the defence against bacterial infections is well demonstrated in neutropenia, an abnormally low number of circulating neutrophils, and a condition that greatly increases the risk for bacterial infections. Both neutrophils and macrophages also help trigger the activation of the adaptive immune system by activating T- and B-cells through the cytokine secretion or direct cell-cell cross talk.

The human immunologic response to bacterial pathogens and sepsis

Upon bacterial invasion, the immune response is initiated when the pattern recognition receptors, such as the toll-like receptors on the surface of the innate immune system cells, identify pathogen-associated molecular patterns[138 139]. This in turn leads to the activation of the innate immunity cell and further secretion of both locally as well as systemically circulating pro-inflammatory cytokines and proteins such as IL-6 and CRP, produced in the liver. These proteins further activate the adaptive immune system and potentiate the innate immune system for the containment and resolution of the infection. However, the activation of the adaptive immune system, specifically naïve T-cells, takes 4-5 days[140], during which the defence against the bacterial pathogens is handled by the innate immune system. During aggressive infections or due to insufficient immunologic response, e.g., caused by immunosuppression, bacteria may reach the systemic circulation causing bacteraemia, which may progress to sepsis[141]. In sepsis, the bacteria multiply in the circulation and initiate an inappropriate hyperinflammatory immune response.

The physiological consequences are significant: extensive peripheral vasodilation and the resulting hypotension, systemic hypoperfusion and tissue hypoxia leading to acidosis and subsequent lactatemia as well as an activation of the coagulation cascade causing thrombosis at a microvascular level (disseminated intravascular coagulation) and bleeding due to consumption coagulopathy. These factors contribute to the development of multi-organ failure, including acute kidney and cardiac injury, resulting in a life-threatening condition. Sepsis has been associated with a staggering in-hospital mortality of 20-30% and long-term mortality of up to 60-80%, even with appropriate treatment at intensive care units[142] [143].

Inflammation

Inflammation, as a term, is used widely and freely in scientific publications, and it is important to distinguish between clinical, subclinical, as well as macroscopic and microscopic types of inflammation. Macroscopic inflammation involves classic, clinical findings visible to the naked eye: *dolor, calor, rubor et tumor* – pain, heat, redness and swelling. These often present as clinical inflammation, i.e., the affected individuals develop symptoms. Microscopic inflammation has been the subject of extensive scientific investigation during the last decades and has been associated to countless phenotypes and diseases. Microscopic inflammation is usually subclinical, i.e., the individuals do not experience any symptoms, and involves subtle changes at tissue or cellular level: vasodilation of the local blood vessels and/or local accumulation of leukocytes that may initiate proinflammatory reactions. Acute inflammation is necessary for the containment of any infection, however, if prolonged or exaggerated, the inflammatory response can become chronic and lead to more harmful than beneficial effects. The length of the inflammation may also depend on the severity of the original stimulus, as sepsis has been shown to cause elevated levels of inflammatory markers even one year after the resolution of the infection[144].

Susceptibility to bacterial infections – environmental and genetic causes

Several diseases, conditions and even treatments, can increase an individual's risk for bacterial infections. The most common causes include different medications and drugs such as immunosuppressive medications, malignancies, chronic ulcers and wounds, chronic pulmonary diseases, alcohol or drug abuse, bone marrow disorders (both congenital and acquired), or the presence of catheters in body cavities, simply to name a few. The effect of weight on the risk of infections seems to be U-shaped, as both underweight as well as obesity are acknowledged risk factors for infections[145]. Smoking increases the risk of infections dramatically and is associated with a two- to four-fold higher risk of contracting pneumonia[146]. Finally, many diseases that affect the quantity and quality of leukocytes, specifically neutrophils, predispose individuals to bacterial infections[147].

In addition to acquired and environmental causes, numerous genetic disorders can result in increased infection susceptibility. Throughout the human evolution, individuals have been in constant interaction

with surrounding micro-organisms, including bacteria and viruses. Infectious diseases have also remained, to this day, one of the most common causes of mortality and morbidity, despite modern technological innovations such as antibiotics and vaccines. Due to this, genetic polymorphisms and mutations causing resistance or susceptibility against microbial pathogen invasions have had a profound effect on the genetic variation in humans[148 149]. Today, it is quite well-known that genetic factors have a great impact on host immunologic defence mechanisms, and several genetic mutations and gene defects that cause inadequate immunologic defences against pathogen invaders have been identified. Such well-recognised genetic disorders include primary immunodeficiencies, also known as human inborn errors of immunity[150]. Primary immunodeficiencies are mostly rare genetic conditions, with varying penetrance and mode of heritance caused by monogenic mutations that result in a gain- or loss-of-function of the gene, altering the transcription of the affected protein. Over 400 gene disorders have been identified to date and the carriers of these genetic mutations are at a high risk of persistent and recurring infections. In addition to infection susceptibility, autoimmune and malignant diseases are also more common in these individuals.

Some studies have also demonstrated how complex, polygenic genotypes are associated with an increased susceptibility or resistance to common infections and that these associations can effectively be investigated using GWAS[151 152]. A GWAS in 2007 demonstrated the potential behind this approach where the authors studied SNPs in less than 500 subjects that were associated with human immunodeficiency virus infection viral load and rate of decline of the CD4 T lymphocyte count[153]. The study identified two SNPs within the class I human leukocyte antigen-region that explained up to 15% of the variation in viral load in the subjects, with seven other SNPs strongly associated with time to disease progression. Following this publication, GWAS has been used to study variants associated with susceptibility to, or protection from human immunodeficiency virus, hepatitis, tuberculosis, and malaria[152]. The discovered loci vary depending on the disease, however, many studies, including a recent comprehensive study by Tian et al. that included 23 GWAS on individuals with European ancestry, have found associations between incidence of infections and variants located in the HLA-region[154 155]. The authors also found that approximately 6% of the variance in the incidence of common infections, of which many bacterial, was attributable to their genome-wide significant loci. Although most studies have identified variants that are associated with susceptibility to the infections, it is important to note also that studies have identified common point mutations that induced a protective role from certain infections as well[156]. Furthermore, although not directly associated to bacterial infections, it is noteworthy that one of the most extensively studied pathogens and its underlying genetic background is candidiasis, and several studies have demonstrated how both monogenic as well as common polygenic mutations can increase the risk of candida-infections[157]. However, despite the efficient and promising methodologic approach, relatively few GWAS on susceptibility to bacterial infections have been reported[158].

2.5. Bacterial lipopolysaccharides

Overview of LPS: Molecular structure and endotoxemia signalling

Bacterial lipopolysaccharides (LPS), also known as endotoxins, are complex glycolipids in the outer layer of the cell wall in gram-negative bacteria. LPS consists of a carbohydrate portion and a lipid A region, which is conserved across many bacterial species[159] (**Fig 2**). The carbohydrate portion is further divided into an inner and outer core oligosaccharide, and a repeating series of distal polysaccharides (also called the O-antigen). Of these components in the LPS-molecules, the lipid A region is the actual endotoxic component that serves as a pathogen-associated molecular pattern and is capable of initiating a comprehensive immunologic reaction in the blood circulation[160]. The presence of LPS within the systemic circulation, is defined as endotoxemia. In endotoxemia signalling, bioactive LPS binds to the LPS-binding protein, further forming a complex with the CD14 receptor[161]. This complex, in turn, acts as a molecule that binds to the pattern recognition sites of the innate immune system, more specifically the toll-like receptor 4 (TLR4). This binding to the TLR4 finally causes the secretion of pro-inflammatory cytokines, namely IL-6, tumour necrosis factor alpha (TNFα) as well as the upregulation of associated inflammatory signalling pathways: the mitogen-activated protein kinase (MAPK)- and the NFκB-pathway[162 163], which gives rise to the inflammatory response. In severe infections by gram-negative pathogens, the endotoxemia is substantial and can induce a hyperinflammatory response, which has long been considered heavily involved in the pathogenesis of sepsis, septic shock, and multiple organ failure[164].

Detoxification and clearance of LPS

LPS is normally present in the gastrointestinal system, particularly the gut, where gram-negative bacterial flora is abundant. A previous, extensive 5-year prospective study, demonstrated that even healthy subjects have measurable levels of endotoxemia[165]. As LPS has the potential to cause devastating inflammation and damage if left unchecked, humans have several methods of detoxifying LPS, both before the translocation of LPS into the circulation as well as afterwards. In the gut lumen on the apical brush border the protein intestinal alkaline phosphatase can detoxify LPS through dephosphorylation[166]. Within the circulation, the enzyme acyloxyacyl hydrolase cleaves secondary fatty acids from the LPS-molecule, thus reducing the toxicity[167]. Furthermore, high-density lipoprotein (HDL) as well as other lipoproteins can neutralize LPS within the circulation by binding to LPS, thereby inhibiting its interaction with TLR-4, and preventing the activation of the inflammation cascade and the subsequent activation of macrophages[168]. This has been clinically observed in endotoxin challenges, in which individuals are given intravenously low doses of endotoxin. In these challenges, individuals with low HDL-concentrations, due to genetic heritance, have had a higher incidence and severity of clinical symptoms as well as more potent inflammatory responses, compared to sex-, age-, and body weight-matched healthy individuals with normal/high HDL cholesterol levels[169]. Administration of

reconstituted HDL to humans during induced endotoxemia has been shown to greatly decrease the production and secretion of several proinflammatory cytokines, therefore blunting the inflammatory response[170]. LPS is also cleared from the circulation by hepatocytes through LDL-receptors[171]. For these reasons and because of the strong link between lipoproteins and LPS, dyslipidaemia has been postulated to increase the endotoxemia-induced inflammatory response during gram-negative infections.

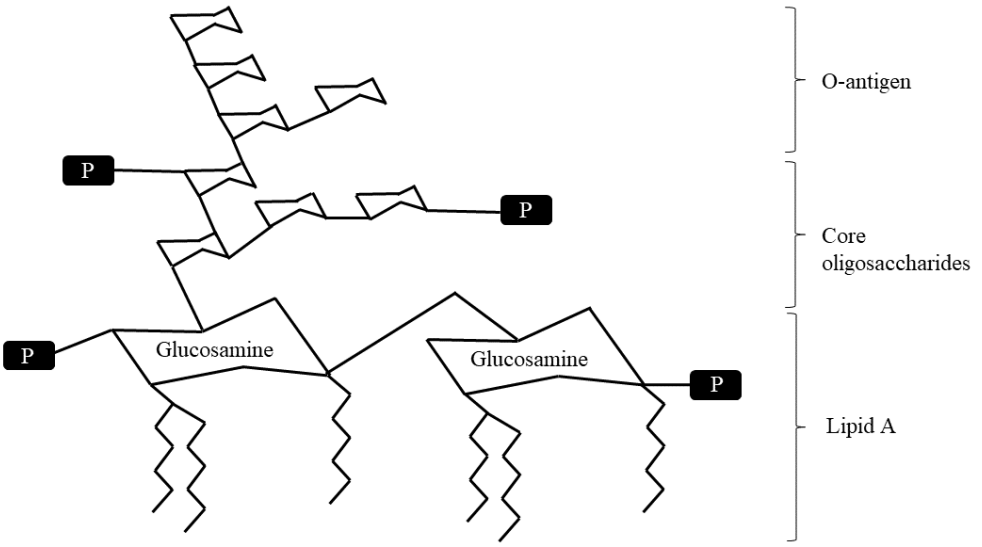


Figure 2. *The typical structure of bacterial lipopolysaccharides (LPS), adapted from How et al.[172]. Lipid A conveys the toxicity of LPS, anchors LPS to the outer layer of gram-negative bacteria and consists of phosphorylated glucosamine saccharides with a varying number of hydrophobic fatty acids. Attached to the lipid A are "core oligosaccharides" with an inner and outer layer, to which a series of repetitive glycan polymers are connected.*

Sources of LPS

LPS can enter the blood circulation through several different anatomical sites, of which the most extensively studied sources of endotoxemia are the oral cavity and gut[173] [174]. In addition to comprehensive bacterial growth, the oral cavity contains numerous superficial blood vessels that may easily bleed, especially during periodontitis, offering ample opportunity for LPS to translocate into the systemic circulation unhindered. In the gut, fats are absorbed as chylomicrons, and lipid-soluble LPS molecules are transported across the epithelial border bound to the chylomicrons, or even passively[175]. Some individuals have also been demonstrated to possess a "leaky gut", i.e., a dysfunctional barrier in the bowels, alleviating the transportation of LPS across the bowel epithelial lining into the circulation

and increasing the levels of endotoxemia[176]. Of note, dysbiosis, disturbance or imbalance in the gut flora, has also been shown to increase LPS-concentrations in the blood, and therefore diets or antibiotic treatments may have a substantial effect on endotoxemia[174 177 178]. Especially high-fat diets have been demonstrated to associate with increased levels of endotoxemia[179]. Moreover, systemic infections (bacteraemia, sepsis) by gram-negative bacteria containing LPS, regardless of infection foci, may cause endotoxemia as well. However, to what extent local infections that do not progress to bacteraemia or sepsis cause endotoxemia, is still unclear.

Endotoxemia, low-grade inflammation and the measurement of LPS

In addition to causing hyperinflammatory responses, subclinical levels of LPS and endotoxemia have been associated with a state of chronic low-grade inflammation[25]. This inflammation has been associated with the development and progression of several metabolic diseases: dyslipidaemia, insulin resistance, the metabolic syndrome, and type 2 diabetes[24 180]. The relationship between obesity and endotoxemia has been frequently researched, and an LPS-dependent mechanism connecting high-fat diet, obesity, and low-grade inflammation has been proposed. According to this theory, a high-fat diet and obesity could cause changes in the microbiome in the gut, leading to alterations in the gut permeability allowing the translocation of LPS into the circulation[175]. The resulting endotoxemia could then promote insulin resistance in the liver, muscle, and adipose tissues through an inflammatory mechanism. However, this chain of causality is yet to be verified.

Levels of endotoxemia, or LPS-activity is traditionally measured using the Limulus amoebocyte lysate (LAL) assay. Although seldom seen in clinical practice, the LAL assay is an established tool in research for the quantitative measurement of endotoxin[181].

2.6 Bacterial infections and diabetes

Diabetes and infections – an overview

Diabetes and hyperglycaemia have generally been considered risk factors for infections in clinical practice for over two decades[182]. The first published observation of an association between infections and diabetes was over a century ago, when Dr Lichty, in 1915, observed several acute infections resulting in death in subjects with diabetes[183]. Bryan et al.[184] showed in 1985 that bacteraemia was twice as common in individuals with diabetes, compared to NDCs and several well-cited works were published in the 1970s and 1980s that demonstrated an impaired leukocyte function in individuals with diabetes, which provided a potential mechanism for the suspicion that individuals with diabetes were more susceptible to infections than the general population[185 186 187]. Epidemiologic evidence of an increased susceptibility to infections in diabetes has been accumulating during the last three decades (**Table 3**). At present, some researchers and clinicians even regard infection susceptibility to be a chronic complication of diabetes. However, although the risk of infections is considered to be elevated

for both type 1 as well as type 2 diabetes, the latter group has been studied much more extensively, while the risk for infections in individuals with type 1 diabetes was, at the time of the start of the present thesis project (March 1st 2010), far from established. It is noteworthy that some infections such as emphysematous pyelonephritis, called 'signal infections', are almost exclusively seen in individuals with diabetes, but the underlying reasons for this are unknown[188 189].

Clinical and epidemiologic studies on diabetes and infections

Previously, studies on the association between infections and diabetes have often either included only individuals with type 2 diabetes, or combined type 1 and type 2 diabetes into joint study cohorts, even though the diabetic phenotype can be considered significantly different between these two forms of diabetes. Only a handful of previous studies have provided some insight into infection frequencies in specifically type 1 diabetes. One study from the Netherlands in 2005 by Muller et al. investigated medical attendance due to certain infections in primary care in individuals with type 1 diabetes, type 2 diabetes, and NDCs[8]. The study demonstrated that individuals with type 1 diabetes had a roughly 1.5-2-fold higher risk for common bacterial infections, compared to NDCs, but, importantly, also a higher risk compared to individuals with type 2 diabetes. After our first publication in 2015, two additional studies have investigated infection frequencies in type 1 and type 2 diabetes separately, and further compared the risk to NDCs[190 191]. Both studies were conducted by the same study group and used comprehensive UK primary care data to assess the infection frequency in individuals with type 1 and type 2 diabetes in the UK. In these studies, they concluded that individuals with diabetes had a higher risk of hospitalisations due to infections, more specifically 3.7-fold higher for individuals with type 1 diabetes, and 1.9-fold higher for individuals with type 2 diabetes. The risk was notably higher (approximately 1.5- to 2-fold) in type 1 diabetes compared to type 2 diabetes, and poor glycaemic control was found to be a powerful risk factor for infection-related hospitalisation. Other epidemiologic studies investigating infection frequencies in type 1 diabetes are scarce. An Australian study that assessed infection-related mortality in individuals with type 1 and type 2 diabetes as well as in non-diabetic individuals, demonstrated an elevated infection-related mortality in type 1 diabetes[192]. However, as the outcome in this study was mortality, the finding itself could not be extrapolated unto infection frequency and the study was unable to conclude whether or not individuals with type 1 diabetes have more frequent infections compared to NDCs or individuals with type 2 diabetes. A drawback of several previous epidemiological studies assessing infection frequencies in individuals with diabetes is that they only used data on infections treated within hospitals, while infection frequencies in outpatient care are much less certain. A recent study from 2018 provided some insight into this in a Canadian cohort, where the authors found a 1.2-fold higher risk of contracting any common infection, both bacterial as well as viral, in individuals with diabetes compared to NDCs, although this study was performed on a mixed cohort with both individuals with type 1 as well as type 2 diabetes[193].

Table 3. Selected previous epidemiologic studies on the prevalence of bacterial infections in type 1 diabetes in chronological order of publication year.

Author	Journal, Year	Study design	Cohort size	Main finding
Muller et al.[8]	Clin Infect Dis, 2005	Prospective cohort study	T1D: n = 705; T2D: n = 6,712; NDCs: 18,911	Individuals with diabetes had greater risk of infections compared with NDCs: lower respiratory tract infection (adjusted odds ratio for T1D: 1.4, for T2D: 1.32), urinary tract infection (T1D: 2.0, T2D: 1.2), bacterial skin and mucous membrane infection (T1D: 1.6, T2D: 1.3), mycotic skin and mucous membrane infection (T1D: NS, T2D: 1.4).
Kornum et al.[7]	Diabetes Care, 2008	Population-based, case-control study	Individuals with first-time pneumonia (n=34,239), of which 101 had T1D and 4,388 had T2D. Control subjects without pneumonia (n=342,390), of which 187 had T1D and 28,299 had T2D	Type 1 and type 2 diabetes were risk factors for pneumonia-related hospitalization (aRRs were 4.4 [3.4-5.8] and 1.2 [1.2-1.3], respectively). The risk was higher with higher HbA_{1c} -levels; HbA_{1c} < 7%: aRR 1.2 (1.1-1.3), HbA_{1c} ≥9%: aRR 1.6(1.4-1.8).
Magliano et al.[192]	Diabetes Care, 2015	Retrospective cohort study	T1D: 85,144 T2D: 1,023,838	Individuals with diabetes had greater infection-related mortality compared to the general population, and the increased risk was greater in T1D (standardized mortality rate 4.4 [95% CI 3.7-5.3]) than in T2D (1.5 [1.4-1.5]).
Critchely et al.[190]	Diabetes Care, 2018	Retrospective cohort study	T1D: 4,496 T2D: 78,964 NDC: 153,341	Poor glycaemic control was powerfully associated with infections. Infection IRRs increased with rising HbA_{1c} compared to NDCs: Optimal control (HbA_{1c} 6-7%) had IRR 1.4 [95% CI 1.4-1.5]) and poor control (≥11%) had 4.7 [4.2-5.2]). This risk was the greatest in subjects with T1D and poor control (IRR 8.5 [5.9-12.2]).
Carey et al.[191]	Diabetes Care, 2018	Retrospective cohort study	T1D: n = 5,863; T2D: n = 96,630; NDCs: 203,518	Individuals with diabetes had higher estimated incidence rate ratio of hospitalisation due to infections compared with non-diabetic controls (3.7 for T1D, 1.9 for T2D). Individuals with T1D had a 2.2-times higher risk of dying from infections compared to individuals with T2D.

T1D indicates Type 1 diabetes; T2D, Type 2 diabetes; NDC, non-diabetic control; NS, non-significant; IRR, incidence rate ratios; aRR, adjusted relative risk.

Although performed on individuals with type 2 diabetes, another study investigating both antibiotic prescription frequencies in outpatient care as well as hospital treated infections, concluded that type 2 diabetes was associated with a 1.2-fold higher use of antibiotics as well as a 1.5-fold higher risk for any hospitalisation due to an infection, compared to NDCs[194]. In addition to an increased risk of contracting an infection, some studies have associated diabetes with increased mortality as well as longer treatment periods in relation to infections[195]. These results, however, have been contradicted by studies finding similar mortality rates in bacteraemia in both diabetic as well as non-diabetic individuals, although in these studies, infection related complications were much more often seen in diabetic individuals[196 197]. A recent comprehensive meta-analysis assessing the incidence of infections in individuals with diabetes estimated that diabetes was associated with a 1.5-2-fold higher risk of contracting common infections when analysing cohort-studies, but up to 2-3-fold higher when analysing case-control studies[198].

Economic aspects

The socioeconomic impact of bacterial infections treated within hospitals has recently been surveyed in the United States[199]. The authors found that out of all hospital visits, infection was the cause of roughly 10% of all hospitalisations of individuals with diabetes. The authors calculated that the aggregate hospital charges covering infection-related hospital visits alone exceeded $48 billion dollars in 2011. Taking further into account the likely absence from work and lost work productivity, these estimates are likely underestimated.

Hypotheses on mechanisms behind the increased risk of infections in diabetes

Although the exact mechanisms through which diabetes induces a susceptibility to infection is still unknown, numerous hypotheses have been formulated. Diabetes seemingly causes changes at both cellular as well as tissue level that increase the risk for infections[200] (**Fig 3**). Firstly, leukocytes, which are essential in the host defence mechanisms against bacterial pathogen invasions, have been shown to function improperly in several regards in diabetes. The leukocytes have a reduced chemotactic ability of adhering to the endothelium and migrating to the inflammation site[201 202]. During infections, neutrophils in diabetic individuals appear to have both an impaired bactericidal activity as well as a reduced phagocytic capability, impeding the initiation of an adequate immunological response as well as the resolution of the infection[203 204]. In experimental animal models, hyperglycaemia has been seen to hamper the mobilisation of neutrophils and impair the activity of the myeloperoxidase enzyme, resulting in lowered neutrophilic phagocytosis and microbial killing abilities[205 206]. In paediatric subjects with diabetes, hyperglycaemia correlated inversely with circulating levels of IgG-antibodies and certain factors of the complement system[207]. Hyperglycaemia also correlates with levels of circulating type 1 interferons and suppresses type I interferon production, consequently also affecting the infection-associated pathways, rendering the individuals more prone to infections[208]. In addition to causing dysfunction of leukocytes, hyperglycaemia and the resulting glycation of proteins in the circulation also

increases the free iron in serum for bacteria, increasing the risk of bacterial infection[209]. In systemic inflammation, hyperglycaemia has been shown to both impair neutrophil activity and inhibit coagulation[210]. Most of the immune system dysfunctionalities associated to diabetes have been discovered in neutrophils or macrophages but interestingly, antibody concentrations from serum have been found to equal the concentrations in non-diabetic subjects in pneumococcal as well as viral vaccine studies[211 212]. This may reflect to some extent a more adequately functioning humoral adaptive immunity in diabetes and has raised the question if the immune system abnormalities in diabetes are mainly attributable to a dysfunctional innate immune system. However, lymphocyte responses to bacterial antigens have also been impaired in diabetes compared to NDCs, potentially reflecting suboptimal humoral adaptive immunologic reactions as well[213].

Not all studies have found leukocyte dysfunction under diabetic circumstances in experimental models. Some diabetic mouse models found that although diabetic mice are increasingly susceptible to bacteraemia caused by gram-negative bacteria, they do not demonstrate an increased bacterial burden compared to non-diabetic mice, to some extent reflecting adequate phagocytosis[214]. In the same study, however, the diabetic mice had substantially higher levels of proinflammatory cytokines in response to the induced infections and lethal hyperinflammatory responses in response to gram-negative bacterial infections. A clinically highly relevant aspect with diabetes and infection frequency is the prevalence and consequences of sepsis in individuals with diabetes. Following the sepsis, the individuals suffer from persistent malfunctioning immunologic reactions, even after initial resolution of the infection[215]. These changes have been hypothesized to result in frequent infections in the following years.

Several factors increase the risk for urinary tract infections in individuals with diabetes, who have frequently been demonstrated to suffer urinary tract infections significantly more often, compared to the general population[216 217]. Bacteria have been shown to have an increased adherence to the epithelial cells in the urinary tract in diabetes. Furthermore, glucosuria, the presence of glucose in urine, has long been proposed to act as a risk factor for both urinary tract infections as well as asymptomatic bacteriuria[218], which in turn is a strong risk factor for a urinary tract infection. However, the evidence behind the association between glucosuria and urinary tract infection is yet controversial[219]. Neuropathic abnormalities such as impaired bladder emptying also increase the risk of urinary tract infections[220].

In addition to cellular risk factors, chronic diabetic complications may also indirectly increase the risk of infections. Both acute as well as chronic foot ulcers are frequent problems for individuals with diabetes. Diabetic neuropathy impairs the function of peripheral nerves causing lowered sensory functions in extremities. This, in turn, predisposes the individuals for foot ulcers that may remain undetected. Poor vascularisation due to peripheral atherosclerosis results in poor blood circulation in the affected area, and subsequently few leukocytes defending against microbial invasion, which

provides an optimal milieu for bacterial colonisation and infection. In general, diabetic wounds develop infection more easily, compared to non-diabetic wounds. Another microvascular diabetic complication indirectly increasing the risk for bacterial infections is diabetic kidney disease (see below).

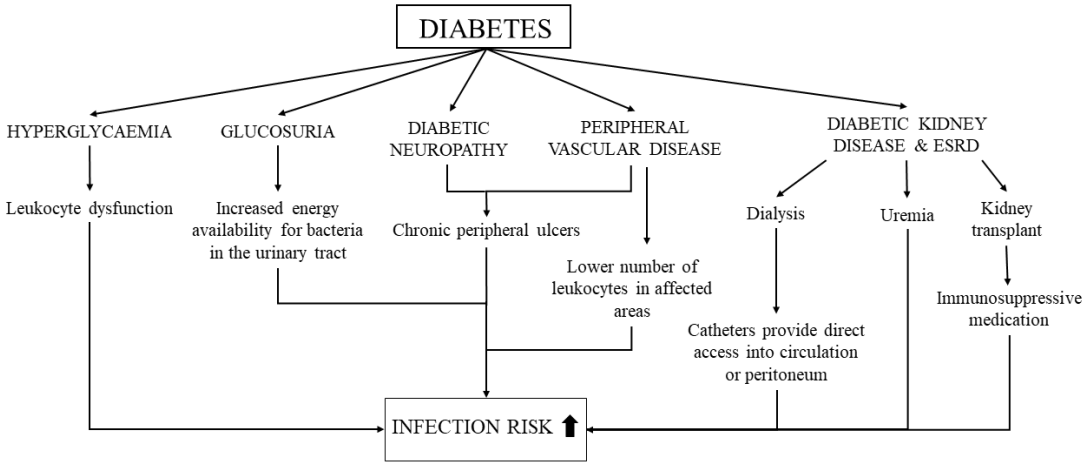


Figure 3. *Hypothesized mechanisms behind increased susceptibility to bacterial infections in diabetes. ESRD indicates end-stage renal disease.*

2.7 Bacterial infections and chronic kidney disease

Bacterial infections as risk factors for kidney failure

The relationship between bacterial infections and kidney diseases is complex and diverse. There are numerous ways for bacterial infections to cause both acute and chronic kidney disease, directly as well as indirectly. Historically, Dr. Bright linked albuminuria with an antecedent infection already in 1836[221]. At present, it's well established that sepsis and associated multi-organ failure are known to cause acute kidney injury, and in critically ill patients, sepsis has been estimated to cause up to 50% of all acute kidney injury cases[222 223]. This kidney injury typically presents as acute tubular necrosis. Even though the individuals may survive the acute kidney injury, up to 40% develop chronic kidney disease afterwards[224]. During urinary tract infections, the infection may spread to the kidney causing pyelonephritis, which may lead to renal scarring and subsequent complications, although this seems to occur mainly in children[225]. Pyelonephritis may also be caused through hematogenous spread from other infections, albeit a far less common aetiology, compared to an ascending urinary tract infection. Bacterial infections can also cause secondary kidney diseases, including nephritic and nephrotic

syndromes, and importantly glomerulonephritis[226]. This effect is caused by infections at other sites inducing the secretion and circulation of immune complexes, which are deposited in the glomeruli causing kidney injury through an immunologic mechanism. Indirectly, bacterial infections are also associated with kidney injury through nephrotoxic antibiotics[227].

Chronic kidney disease as a risk factor for bacterial infections

Whether the opposite association – if chronic kidney disease increases the risk of infection – holds true, is unclear and depends on the severity of the associated impairment in renal function. More specifically, ESRD has been found to increase the risk of bacteraemia and sepsis substantially[228 229]. Regardless of dialysis method (intraperitoneal- or haemodialysis), bacteria are repeatedly offered a direct route into the peritoneum or circulation during dialysis, consequently increasing the risk for severe bacterial infections, such as peritonitis or bacteraemia. Individuals with ESRD who have received a kidney transplant are continuously on immunosuppressive medication for the prevention of host versus graft complications, which increases the risk of infections[230]. Uraemia, a condition arising during ESRD as end-products of metabolism are insufficiently cleared from the circulation by the kidneys, has also been known to predispose the individuals to infections[231]. However, the magnitude of the infection frequency attributable to chronic kidney disease and not to the underlying disease that caused the kidney impairment is unclear, as most studies assessing infection frequencies in ESRD have pooled the individuals into one cohort, regardless of the aetiology behind the kidney disease. As diabetes even without kidney disease has an impact on infection susceptibility and is also the most common cause of ESRD, the pooling potentially confounds the results and dilutes the results for individuals with diabetes, while exaggerating the results for individuals with ESRD due to causes other than diabetes.

Regarding research on whether milder forms of chronic kidney disease, in addition to ESRD, increase the risk of infections, the connection is far from established and publications are fewer. Wang et al. demonstrated, in 2011, that individuals with chronic kidney disease, and an eGFR below 60 ml/min, had an increased risk of infection related mortality[232]. However, since the study assessed infection-related mortality, the results cannot be said to have demonstrated an increased susceptibility to infections or infection frequency in individuals with chronic kidney disease. A more recent systematic review assessed community-acquired infection frequencies in individuals with and without pre-dialysis chronic kidney disease in 14 eligible studies and found that chronic kidney disease seemed to be associated with an increased frequency of infections[233]. However, the authors also found that in the included studies, confounding factors were poorly adjusted for, and the grading of the kidney disease was inaccurate, making the interpretation of the results challenging.

One important aspect in the relationship between bacterial infections and the development of chronic kidney disease are endotoxins. The nephrotoxic abilities of LPS are well known, and LPS has long been used in experimental studies to cause acute kidney injury and septic shock in mice, dose-dependently.

Although the mechanisms through which LPS causes kidney injury is still unknown, it has been hypothesized that the damage could involve mitochondrial injury and oxidative stress, as histopathological findings in the rat kidney after LPS-treatment resemble those that develop after exposure to hypoxia[234] [235]. This may be mediated through an interleukin-18 (interferon-γ) signalling pathway[236]. In addition to its well-established role as a cause of acute kidney injury, LPS and endotoxemia have also been linked to chronic kidney disease, and, specifically diabetic kidney disease. LPS-activity has been shown to correlate with the severity of diabetic kidney disease as well as serve as an independent risk factor for the progression of diabetic kidney disease[23]. The elevated levels of LPS in pre-dialysis chronic kidney disease also seem tightly connected to a disturbed gut flora in type 2 diabetes[237]. Following this trail of thought, it has also been postulated that gastrointestinal dysfunction and diseases may increase the risk of chronic kidney disease[166].

Surprisingly, even though there are several possible ways for bacterial infections and diabetic kidney disease to associate with one another, very few studies have assessed this association in epidemiologic study settings, especially in pre-dialysis individuals or in milder forms of diabetic kidney disease. Hence, epidemiologic studies assessing the association between bacterial infections and the different stages of diabetic and other forms of chronic kidney disease are sorely needed.

2.8 Bacterial infections and cardiovascular disease

The role of infections in the pathophysiologic processes of cardiovascular disease has been extensively studied over the last three decades, however the causal link between the two is yet unclear. Infections have long been considered to instigate and accelerate events in the pathophysiology of atherosclerosis[20] [238] [239]. This hypothesis was originally published over 40 years ago, in a series of experimental studies where viral infections in chickens were seen to cause atherosclerosis and the injection of viruses into smooth muscle cells resulted in the accumulation of cholesterol and lipids[240] [241]. Since then, several hypotheses as to how infections could contribute to atherosclerosis have been postulated.

Proposed mechanisms through which infections could contribute to cardiovascular disease

Some studies have proposed that the infective pathogens could directly promote atherogenesis by invading endothelial cells in the intima, where they could stimulate the inflammatory response locally, and thereby promote the development of the atherosclerotic lesion. This theory is supported by studies that have demonstrated the presence of several bacterial and viral pathogens within human atherosclerotic plaques[239]. The most notable bacterial pathogens include: *Chlamydia pneumoniae, Mycoplasma pneumoniae, Helicobacter pylori,* and *Poryphyromonas gingivalis*[242] [243]. The presence of these bacteria within the atherosclerotic plaques has mainly been detected by nucleic acid or antigens, however, *C. pneumoniae* has also been cultured from atherosclerotic arteries[244]. Experimental studies have demonstrated how *C. pneumoniae* has a direct proatherogenic effect in the intima, although this effect has solely been seen in hyperlipidaemic animal model studies[239]. Additionally, multiple viruses

have been likewise detected in atherosclerotic plaques, especially viruses belonging to the *Herpes*-family[239]. Infections by several different pathogens seem to have an additive role in the risk of atherogenesis, demonstrated by a study where seropositivity to certain microbial pathogens (*Cytomegalovirus*, *C. pneumoniae*, *Hepatitis A virus*, *Herpes Simplex virus type 1 and 2*) was strongly associated with coronary artery disease[245]. An increasing aggregate number of seropositivity of the infections, reflecting a total pathogen burden, was also seen to strongly associate with an increased risk of coronary artery disease.

One mechanism through which gram-negative pathogens could increase the risk of cardiovascular disease is LPS. Endotoxemia has been strongly associated to cardiovascular disease and specifically, coronary heart disease[24 25 246 247 248]. LPS can activate the TLRs of the endothelium in the intima, leading to the recruitment of leukocytes to the site and the initiation of the inflammatory cascade observed in atherosclerosis[249]. As LPS has a direct, independent nephrotoxic effect, it is also possible that LPS damages cardiac cells directly in a corresponding manner. LPS could also act as a causal factor in atherosclerosis indirectly by increasing levels of systemic inflammation. Another possibility is that the effect of LPS on cardiovascular disease is mediated through the increased risk of kidney disease attributable to LPS, as chronic kidney disease is a major risk factor for cardiovascular disease. Of note, several of the studies demonstrating an association between LPS and cardiovascular disease did not adjust their models for kidney function or failure. Although several hypotheses have been proposed, to date, it has been difficult to assess the chronology of the disorders, and it is unclear if endotoxemia precedes cardiovascular disease, or if the gut dysbiosis and resulting endotoxemia follows cardiovascular disease[250]. Cardiovascular disease might cause congestion in the veins in the mesenteric circulation, leading to increased bowel permeability and translocation of LPS into the circulation[246]. It is also noteworthy that individuals with diabetes have higher levels of endotoxemia, compared to healthy individuals from the general population, theoretically putting them at an even greater risk for atherosclerosis and cardiovascular disease through LPS.

When assessing infections as risk factors for cardiovascular disease, it is also important to distinguish between the pathophysiologic events in slowly developing, chronic cardiovascular disease, mostly meaning atherosclerosis, as opposed to acute cardiovascular disease events. Severe acute infections predispose individuals to incident cardiovascular disease[21]. Acute coronary events are often preceded by a systemic inflammatory burst that has been thought to act as a trigger for acute coronary syndromes[251 252]. This inflammatory burst, could among other stimuli, be caused by bacterial invasion and infection. Furthermore, pneumonia can cause hypoxia, putting greater stress on the myocardium in individuals with existing coronary heart disease, thus, trigger an acute coronary event[253 254]. In sepsis, myocardial damage is common, and individuals are at an increased risk for acute coronary syndromes, both during the infection but also afterwards, years after the resolution of the infection[255]. This has largely been explained by the presence of systemic lactatemia and hypoxia, in addition to the

prothrombotic state coagulation disorders, the inflammatory state as well as the endothelial dysfunction in the vasculature, present during sepsis. This extended post-infectious elevated risk of cardiovascular disease also applies to pneumonia.

Finally, previous research has proposed that infections could advance atherosclerosis indirectly, through the effects of systemically circulating pro-inflammatory cytokines[20]. This could be achieved by local, peripheral infections causing the upregulation and secretion of cytokines and a systemic inflammatory response, which may promote local inflammation in atherosclerotic plaques[20].

Scientific evidence contradicting the infection-atherosclerosis hypothesis

Research disputing the role of infections as risk factors for cardiovascular disease with atherogenic aetiologies, is largely based on a series of randomized clinical trials performed in the late 20th to the beginning of the 21st century[256 257 258 259 260]. In these trials, individuals with established atherosclerosis were administered either a placebo or differing doses of azithromycin or other macrolides for varying follow-up periods, ranging from three months to two years, for the prevention of coronary heart events. Although early pilot studies showed a reduction of cardiovascular events after azithromycin administration in individuals with a previous myocardial infarction and high titres of *Chlamydia pneumoniae* IgG-antibodies, these large-scale randomized trials consistently failed to demonstrate protective effects of macrolide therapies for cardiovascular disease events. Similar results were also observed in later meta-analyses on the effect of azithromycin in the prevention of cardiovascular disease events[261]. Interestingly, the STAMINA trial differed to the other previous trials in regard to both results as well as the antibiotic intervention regimen[262]. The authors of this trial found that in individuals newly admitted to hospitals for acute coronary syndromes, a one-week course of either amoxicillin together with both metronidazole and omeprazole, or azithromycin together with metronidazole and omeprazole reduced the risk of future cardiac events by 36%, as compared to placebo, until the end of the one year follow-up. Notably, no differences in outcomes were observed between the two different antibiotic interventions and no changes in *Chlamydia pneumoniae* or *Helicobacter pylori* antibody statuses were related to the outcomes.

Regardless of the study outcomes and findings, certain limitations must be addressed in conjunction with the discussion of the results in these randomized trials. The study subjects were individuals with existing extensive atherosclerosis and established coronary heart disease, and several studies only included individuals that tested seropositive for *Chlamydia pneumoniae*, making the extrapolation of the results unto other groups of individuals challenging. Some trials were inconclusive due to statistical power issues and small sample sizes, even though results seemed to indicate some protective effect of macrolides[263]. The antibiotic regimens used in the studies also varied greatly, and the length of follow-up was usually less than one year, while the longest trial followed the subjects for up to two years. As atherosclerosis is a disease progressing over the course of several years, the follow-up times were quite

short when considering the usual progression of the disease. Finally, excluding the STAMINA trial, the only antibiotics used in the trials were macrolides. Although their penetrance into atherosclerotic plaques has been well-documented[264], macrolide-resistant bacteria have been steadily increasing during the last 20 years, world-wide, and during the trials in 2005, up to 30% of *Streptococcus pneumoniae* were resistant to macrolides in the US, with similar numbers globally[265]. Although the studies' rationale was the treatment of potential *Chlamydia pneumoniae* colonisation, antibiotic resistance is an important factor in monotherapy and was not addressed in the trials. Macrolide resistant bacteria has seen a large growth since the start of the 21st century. Macrolides are no longer recommended as monotherapy for pneumonia in Finland, mostly due to the emergence of macrolide-resistant *Streptococcus Pneumoniae* strains[266]. Further, the trials mainly sought to treat chronic infection, although acute infections are important risk factors for incident cardiovascular disease. To summarize, these clinical trials are unable to conclude whether antibiotic treatments affect the earlier stages of atherogenesis, or if they increase or decrease the risk of acute coronary syndromes in the absence of previous coronary heart disease. Furthermore, the majority of the trials do not elucidate whether other antibiotics covering other bacterium spectra outside of the macrolide-range affect atherogenesis or the risk of cardiovascular disease. Although macrolide therapy is considered effective for the treatment of infections by *Chlamydia pneumonia,* the effect of these antibiotics on other gram-negative bacteria, such as *Escherichia coli* is weak. The fact that LPS is only found on gram-negative bacteria and is a potential link between bacteria, infections, and cardiovascular disease is of importance when investigating infections as risk factors for cardiovascular disease. Hence, the results from these trials can hardly be characterized as conclusive or said to rule out infections as causal factors in atherosclerotic or cardiovascular disease.

2.9 Bacterial infections and diabetic retinopathy

Although inflammation has been hypothesized as an important pathophysiologic event in the development of diabetic retinopathy and previous research has demonstrated a potential association between infections and both micro- and macrovascular complications, very few studies have assessed the relationship between infections and diabetic retinopathy. In non-diabetic individuals, systemic infections have been shown to cause retinal auto-immune diseases, demonstrating the close relationship between the eye and the immune system[267]. Epidemiologic or clinical studies regarding infections and diabetic retinopathy are lacking, but some experimental studies have demonstrated interesting results, as treatment with tetracyclines has alleviated the production of inflammatory cytokines in the eye in diabetic rats[268]. A recent small-scale open-label clinical trial showed that minocycline improved visual acuity and reduced central macular thickness and volume in individuals with diabetic macular oedema[269]. The macrolide rapamycin reduced cell-proliferation and angiogenesis in the retina, and in phase -I clinical open-label clinical trials, local single injections of the drug into the eye improved visual acuity and decreased retinal thickness in type 1 and type 2 diabetes[270 271]. These drugs are thought to

alleviate diabetic retinopathy by suppressing the inflammatory activity locally in the eye, however, the more specific causality behind this association is unclear[270]. None the less, the hypothesis that bacterial infections could associate with diabetic retinopathy is further strengthened, as antibiotics seem to have a beneficial effect on the severity of diabetic retinopathy.

2.10 The unanswered question – The relationship between diabetes, infections and the chronic complications of diabetes

Substantial scientific evidence has linked inflammation to the development and progression of chronic diabetic complications. Compared with the general population, individuals with diabetes are more prone to bacterial infections, which if severe, can cause increased inflammatory states lasting years after the resolution of the infections. The infections have further been hypothesized to act as important risk factors or even as causal factors in atherosclerosis and have been frequently associated with cardiovascular disease as well as acute and chronic kidney disease in the general population. Additionally, LPS has been associated with cardiovascular disease in the general population and the development and progression of diabetic kidney disease.

Despite previous research and known associations, the relationship between bacterial infections and cardiovascular disease, diabetic kidney disease, and diabetic retinopathy is yet to be investigated. Studies assessing the prevalence of bacterial infections in individuals with specifically type 1 diabetes are lacking. Finally, the mechanisms, specifically potential genetic factors contributing to an increased susceptibility to bacterial infections in diabetes, are unknown.

3. AIMS OF THE THESIS

I. Survey the prevalence of bacterial infections in individuals with type 1 diabetes compared with the general population.

II. Examine the relationship between glycaemic control and infection frequency in individuals with type 1 diabetes.

III. Assess the association between bacterial infections and diabetic kidney disease in individuals with type 1 diabetes.

IV. Evaluate the association between bacterial infections and the risk of coronary heart disease in individuals with type 1 diabetes.

V. Investigate the association between bacterial infections and diabetic retinopathy in type 1 diabetes.

VI. Explore genetic factors affecting the susceptibility to bacterial infections in individuals with diabetes.

4. SUBJECTS, MATERIALS AND METHODS

4.1 The FinnDiane Study

All four publications presented in this thesis involved the study of participants from the Finnish Diabetic Nephropathy Study (FinnDiane, www.finndiane.fi). The FinnDiane Study is an ongoing prospective follow-up nation-wide multicentre study, founded in 1997, with the aim to research and identify key risk factors for the development and progression of diabetic complications, with a special emphasis on diabetic kidney disease. The study is conducted all over Finland and the residences of the participants closely follow the distribution of the Finnish general population (**Fig 4**). This minimises sampling bias according to the geographic location and the selection of participants. The study is conducted at over 90 centres in Finland, including all five university central hospitals, all 16 central hospitals, close to 30 regional hospitals, and over 30 major primary healthcare centres. As all adult individuals with type 1 diabetes at these centres are invited to participate in the FinnDiane Study, the recruitment strategy is random, which further minimises potential sampling bias.

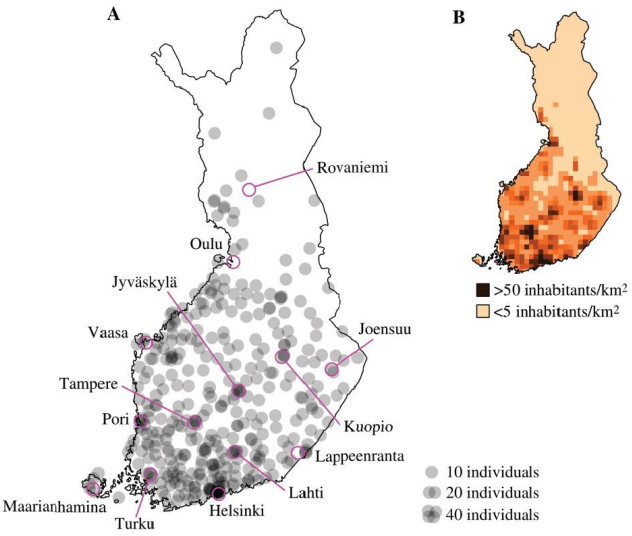


Figure 4. *A) The geographical locations of the FinnDiane study centres and B) the distribution of the population of Finland. Modified from* [272] *and published with the approval of the original author.*

At the baseline visit, study participants undergo a medical examination and the attending physician fills a standardized questionnaire. The presence of existing diabetic complications, medications, and other diseases is recorded. Clinical measurements are taken (**Table 4**), including anthropometric

measurements, blood pressure, and an ECG. In addition to the clinical measurements, fasting blood samples are collected, for the measurement of several parameters, including lipids, HbA_{1c}, and importantly, DNA-samples. Twenty-four-hour urine samples are also collected for the assessment of albuminuria and the development or progression of diabetic kidney disease. After the baseline visit, the participants are prospectively followed with similar study visits with three to four-year intervals. Furthermore, medical data is actively collected from all study centres, including blood and urinary samples taken between the study visits in conjunction with other medical visits, as well as fundus photographs of the participants' retinas. To this date, FinnDiane has collected comprehensive and prospective medical data on over 5400 individuals with type 1 diabetes, and the median follow-up of the participants is 15 years. This has resulted in one of the most extensively researched and well-defined cohorts of individuals with type 1 diabetes in the world, offering unique research possibilities.

Table 4. Clinical measurements obtained during FinnDiane-study visits.

Clinical	Glucose
	Blood pressure
	Height
	Weight
	ECG
	Applanation Tonometry
Blood analysis	Blood cell count
	Total cholesterol
	HDL
	Triglycerides
	HbA_{1c}
	Creatinine and eGFR
	Vitamin D
	IL-6
	CRP
	Glucose
	Sodium, Potassium, Creatinine
	Phosphate
	Parathyroid hormone
	DNA-sample
Urine analysis	Urinary albumin
	Urinary creatinine

ECG indicates electrocardiogram; HDL, high-density lipoprotein; HbA_{1c}, glycated haemoglobin; eGFR, estimated glomerular filtration rate; IL-6, Interleukin 6; CRP, C-reactive Protein; and PTH, Parathyroid-hormone.

4.2 Clinical characteristics of the study populations

The FinnDiane study participants

From the FinnDiane study cohort, subsets were designed for studies I-IV according to the different phenotypes explored in the studies: diabetic kidney disease, cardiovascular disease, diabetic retinopathy and finally, genetic data (**Table 5**).

Table 5. The baseline clinical characteristics of all FinnDiane participants in 2019, stratified by the severity of diabetic kidney disease.

Variable	Normal AER	Microalbuminuria	Macroalbuminuria	ESRD
N	3,330 (65.0)	641 (12.5)	712 (13.9)	443 (8.6)
Sex (female %)	1751 (52.6)	275 (42.9)	278 (39.0)	166 (37.5)
Age (years)	36.2 (26.8-46.5)	39.6 (30.2-49.8)	42.1 (34.0-50.3)	44.9 (39.3-51.1)
Age at onset of diabetes (years)	16.9 (10.7-26.5)	11.8 (7.1-19.1)	11.8 (7.6-17.4)	11.6 (7.0-16.0)
HbA_1c (mmol/mol)	66 ± 16	73 ± 16	75 ± 17	69 ± 17
Office systolic blood pressure (mmHg)	130 ± 16	137 ± 17	146 ± 21	152 ± 24
Office diastolic blood pressure (mmHg)	78 ± 9	80 ± 10	82 ± 11	84 ± 13
Waist-hip-ratio	0.85 ± 0.08	0.88 ± 0.08	0.90 ± 0.09	0.93 ± 0.10
Body Mass Index (kg/m^2)	24.6 (22.6-26.8)	25.5 (23.1-28.1)	25.6 (23.3-28.6)	23.8 (21.3-26.3)
eGFR (mL/min/1.73 m2)	106 (93-118)	100 (82-113)	58 (32-87)	NA
History of smoking, n (%)	1309 (42)	316 (51)	409 (60)	241 (60)
LDL-cholesterol (mmol/l)	2.89 ± 0.82	3.04 ± 0.84	3.30 ± 1.00	2.99 ± 1.16
Triglycerides (mmol/l)	1.12 ± 0.70	1.36 ± 0.92	1.75 ± 1.17	1.63 ± 0.89
HDL-cholesterol (mmol/l)	1.39 ± 0.39	1.33 ± 0.40	1.22 ± 0.39	1.22 ± 0.42
Antihypertensive medication, n (%)	553 (16.7)	438 (69.0)	672 (95.6)	394 (91.0)
Lipid-lowering medication, n (%)	296 (8.9)	99 (15.4)	209 (29.4)	171 (38.6)
History of retinal laser treatment, n (%)	444 (13.3)	308 (48.1)	552 (77.5)	409 (92.3)
Coronary heart disease, n (%)	113 (3.4)	40 (6.2)	84 (11.8)	109 (24.6)

AER indicates albumin excretion rate; NA, Not Applicable; HbA_{1c}, glycated haemoglobin; eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; LDL, low-density lipoprotein; and HDL, high-density lipoprotein. Data is presented as means ± standard deviation, median (interquartile range) or percentages as appropriate.

Study IV: The DIREVA cohort

In study IV, in collaboration with the Botnia study group, we included participants from the Diabetes Register Vaasa (DIREVA) study. The DIREVA study was founded in 2007, as a long-term prospective follow-up study of individuals with diabetes in the Vaasa region in Finland, with the goal to improve the individual treatment of diabetes. In the beginning of 2019, the study included up to 7000 individuals with diabetes. The DIREVA study is comprised of individuals with several kinds of diabetes: MODY, LADA, type 1, and type 2 diabetes. From this cohort, a subgroup with available genetic data was included (n=4,247).

Table 6. The clinical characteristics of the DIREVA individuals included in study IV.

Parameter	DIREVA-cohort
n	4,247
Sex, n (% female)	1,868 (44.0)
Average age during follow-up (years)	63.9 (54.6-71.4)
Average HbA_{1c} during follow-up (mmol/mol)	51.7 ± 12.6
Age at onset of diabetes (years)	57.0 (46.0-64.8)
Systolic blood pressure (mmHg)	140 (130-150)
Diastolic blood pressure (mmHg)	80 (74-87)
eGFR (ml/min/1.73m^2)	81.5 (63.7-94.6)
LDL cholesterol (mmol/L)	3.1 (2.5-3.8)
Subjects treated with exogen insulin, n (%)	1.726 (40.6)
Proportion of T1D (%)	545 (12.8)
Follow-up (years)	11.6 (7.1-18.3)

HbA_{1c} indicates glycated haemoglobin; eGFR, estimated glomerular filtration rate; LDL, low-density lipoprotein; and T1D, Type 1 diabetes. Data is presented as means ± standard deviation, median (interquartile range), or percentages, where appropriate.

4.3 Finnish nationwide registers

Finland offers unique observational and epidemiologic research opportunities due to nationwide registers upheld by governmental faculties and branches. The registers are mandatorily used across the country in clinical care and include data on hospital treatments, drug prescription purchases, and death certificates. The register data are freely available from the government upon reasonable researcher request through written applications. At birth or acceptance of citizenship, each Finnish citizen is provided with a unique social security number. The register records all data using this social security number, improving the register's accuracy and comprehensiveness. The use of these national registers allowed us to effectively and retrospectively acquire data on bacterial infections treated both outside

and within hospitals over extensive follow-up periods. Additionally, data on comorbidities, the presence and timing of the development of diabetic complications, and death were collected from the register data.

The Finnish National Drug Prescription Register – Data on bacterial infections treated in outpatient care.

Since 1994, The Social Insurance Institution in Finland has upheld the Finnish National Drug Prescription Register. This register contains nation-wide information on all drug prescription purchases from pharmacies for every Finnish citizen with a social security number. The register records the date of the purchase, the Anatomical Chemical Classification System (ATC-) code of the drug, the number of packages, and the size of each package. The ATC-system is a classification system constructed and used by the World Health Organization (WHO) to classify medicinal drugs according to their therapeutic use or indication. The class of each drug in the system contains 5 levels of information, with each consecutive level more specific than the former. The first level indicates the main groups of diseases the drug is used to treat, the second level indicates the therapeutic subgroup, the third level indicates the pharmacological subgroup, the fourth level indicates the chemical subgroup and the final fifth level indicates the specific compound (**Fig 5**).

J01CA04 Amoxicillin

Level 1 (Anatomical main group):	**J** – Anti-infectives for systemic use
Level 2 (Therapeutic subgroup):	**J01** – Antibacterials for systemic use
Level 3 (Pharmacological subgroup):	**J01C** – Beta-lactam antibacterials, penicillins
Level 4 (Chemical subgroup):	**J01CA** – Penicillins with extended spectrum
Level 5 (Specific substance):	**J01CA04** – Amoxicillin

Figure 5. *An example of the drug amoxicillin to illustrate the structure and practical use of the ATC-code.*

In Finland, depending on the drug and disease requiring the treatment, the individual is entitled to a varying amount of state reimbursement. The Basic Refund Category refunds 40% (e.g., medication for hypertension), the Lower Special Refund Category refunds 65% (e.g., anticoagulation medications in the treatment of atrial fibrillation) and the Higher Special Refund Category refunds 100% (e.g., insulin in the treatment of diabetes) of the price of the drug. Special or full reimbursement of a prescription purchase requires certificates, sent by the physician to the Social Insurance Institution, ensuring that the individual fulfils the criteria for special reimbursement. The diagnoses and data within the certificates

are also available in the drug prescription register and provide additional information on comorbidities of the individuals, for example, the presence of diabetes requiring insulin treatment, chronic pulmonary disorders such as asthma, or malignant diseases.

In studies I-IV, we used codes under the subcategory J01 **(Table 6A)** representing all oral systemic antibiotic drugs to investigate antibiotic purchases by the subjects. In Finland, as opposed to several other countries, systemic antibiotics can't be purchased without a prescription written by a medical professional. Therefore, the antibiotic purchase seen in the drug prescription register reflects a bacterial infection necessitating an antibiotic treatment diagnosed by a health professional in outpatient care. The register, however, does not contain information on antibiotics purchased abroad or antibiotics used within hospitals, although data on bacterial infections treated within hospitals is available in the Finnish Hospital Discharge Register.

The drug prescription register does not include the indication for which the drugs are prescribed. Due to this, for most of the prescription purchase events it is challenging to ascertain the bacteria or infection foci for which the antibiotic was prescribed. However, an exception to this are the antibiotics prescribed for urinary tract infections. The national guidelines for the treatment of urinary tract infections have not undergone substantial changes during the last three decades, and the antibiotics used in the treatment of urinary tract infections are furthermore quite specific. Therefore, certain antibiotics and their respective prescription purchases in the drug prescription register, can be considered to mainly reflect urinary tract infections (**Table 6B**).

Table 6. A. The sub-categories of the antibiotics in the J01 category in the Anatomical Chemical Classification System (ATC). B. The antibiotics primarily used in the treatment of urinary tract infections.

A.

ATC-code (Level 1-3)	Therapeutic and pharmacological subgroup of the class of antibiotics
J01A	Tetracyclines
J01B	Amphenicols
J01C	Beta-lactam antibacterials, Penicillins
J01D	Other Beta-lactam antibacterials
J01E	Sulfonamides and Trimethoprim
J01F	Macrolides, Lincosamides and streptogramins
J01G	Aminoglycoside antibacterials
J01M	Quinolone antibacterials
J01R	Combinations of antibacterials
J01X	Other antibacterials

B.

ATC-code (Level 1-5)	Therapeutic and pharmacological subgroup of the class of antibiotics
J01CA08	Pivmecillinam
J01EA01	Trimethoprim
J01EC02	Sulfadiazine
J01EC20	Combinations
J01EE01	Sulfamethoxazole and trimethoprim
J01EE02	Sulfadiazine and trimethoprim
J01MA01	Ofloxacin
J01MA02	Ciprofloxacine
J01MA06	Norfloxacine
J01MA12	Levofloxacine
J01XE01	Nitrofurantoin
J01XE51	Nitrofurantoin, combinations

The Care Register for Health Care - Bacterial infections treated within hospitals.

The Care Register for Health Care, also named the Finnish Hospital Discharge Register, was founded in 1969 by the National Institute for Health and Welfare (THL). The register contains information on all hospital visits in Finland for every Finnish citizen with a social security number. The register stores information on admission date, discharge date, and the diagnoses of the hospitalized individual, including diagnoses on both chronic as well as acute diseases emerging before and during the hospital visit. As of 1994, the register also records data on potential surgical procedures performed both within as well as outside of hospitals in the private sector. The diagnoses are recorded using the International Classification of Diseases (ICD-) system, implemented, revised, and maintained by the World Health Organization (WHO). Surgical procedures are recorded based on the Nordic Medico-Statistical Committee (NOMESCO) classification system. Using the ICD-codes, we were able to identify specifically bacterial infections that were treated within hospitals (**Table 7**).

Causes of Death Register

As of 1936, death certificates have been implemented as a nation-wide standard procedure and are routinely written for each death, by a physician. The Cause of Death Register was created the same year and has, since then, recorded the primary cause of death for each individual in Finland. Since 1996, the ICD-codes (10^{th} revision) have been included. The Cause of Death register is maintained by Statistics Finland, a governmental agency.

Table 7. Classification of bacterial infections according to the International Classification of Diseases (ICD), 10th revision.

ICD-Code	Specification of infection foci
A00-A05	Intestinal infectious diseases
A15-A19	Tuberculosis
A40-A41	Sepsis
A51, A53.9, A54-58, N41, N43.1, N45, N48.1, N70-72, N73.3-73.5, N73.8-73.9, N75.1, N75.8, N76.4	Infections with a predominantly sexual mode of transmission, male genital infections, and female pelvic organ infections
D73.3, E32.1, K75.0, K61, K63.0	Abscesses
G00, G04.2, G06, G08	Central nervous system infections
H04.3, H05.0, H44.0	Eye infections
H60-60.1, H66.0-66.4	Ear infections
I33, I38	Heart infections
J01, J02.0, J03.0, J32, J36	Upper respiratory tract infections
J13-15, J16.0, J18.9, J20.0-J20.2, J85, J86	Lower respiratory infections
K04.0-04.7, K05.0, K0.5.2-K05.3, K10.2-10.3, K11.3, K12.2	Infections of oral cavity, salivary glands and jaw
K35-37	Appendicitis
K65.0, K65.9	Peritonitis
A46, L00-05, L08.0-L08.1, L73.2	Infections of the skin and subcutaneous tissue
M00, M46-46.5, M60.0, M65.0-65.1, M71.0-71.1, M72.6, M72.8, M86	Osteomyelitis, infections of joints, muscles, and fascia
N10-N11.1, N13.6, N15.1, N15.9, N30.0, N30.8-30.9, N34.0-34.1, N36.2, N39.0	Renal tubulo-interstitial infections and urinary tract infections
A20-28, A30-32, A36-39, A42-45, A48-49, A65-79, B95-96, B98.1, E06.0, I00-02	Other bacterial diseases

4.4 Study designs and cohorts

The study design of publications I-III were all register-based prospective follow-up studies, where we used register data to investigate the association between bacterial infections and certain chronic complications of diabetes. All three studies included the FinnDiane study participants, from which individuals were selected based on certain inclusion and exclusion criteria (**Table 8**). For all studies, type 1 diabetes was defined as age at onset of diabetes <40 years with permanent insulin treatment started within one year after the diagnosis of diabetes. All individuals included in the FinnDiane study are adults, with an age ranging from 18 to 65 years at baseline.

Study I aim, cohort, and phenotype

In this study, our aim was to survey the prevalence of bacterial infection frequencies in individuals with type 1 diabetes compared with the general population, as well as study the association between bacterial infection frequencies and diabetic kidney disease. This study included all individuals with type 1 diabetes, for whom we could, at the time, identify three age- and sex-matched NDCs from the Finnish Public Register Centre (n=4,748 and n=12,954 respectively). To further investigate the infection frequencies at different stages of diabetic kidney disease, we identified individuals from the main cohort with an ascertained stage of diabetic kidney disease at baseline (n=4,169). As the stage of diabetic kidney disease advanced in some individuals during the follow-up, we determined the stage of diabetic kidney disease for each subject for each year of follow-up and calculated the total numbers of follow-up years for each stage of diabetic kidney disease. Each subject participated with their follow-up years to each corresponding kidney disease stage at which the subject had been during each year. Data on antibiotic purchases and hospitalisations due to bacterial infections were collected from the Finnish National Drug Prescription Register and the Finnish Hospital Discharge Register, respectively.

Study II aim, cohort, and phenotype

In study II we investigated the association between bacterial infections and incident coronary heart disease in type 1 diabetes. Coronary heart disease events were defined as severe events: death or hospitalization due to myocardial infarction, treatment with coronary bypass surgery or percutaneous coronary intervention. Follow-up started at baseline and ended in death, a coronary heart disease event, or at the end of the period of available register data (Dec 31st 2015). As the presence or progression of diabetic kidney disease is a major risk factor for coronary heart disease, we only included FinnDiane participants with an ascertained stage of diabetic kidney disease at baseline and for whom no progression of diabetic kidney disease had been observed during follow-up (n=3,781). Individuals with a history of coronary heart events at baseline as well as individuals who developed ESRD at any point during follow-up were excluded. Data on bacterial infections were collected from the Finnish National Drug Prescription Register and data on coronary heart disease from the Finnish Hospital Discharge Register as well as the Causes of Death Register.

Study III aim, cohort, and phenotype

In study III we investigated the association between bacterial infections and severe diabetic retinopathy. We compared antibiotic purchase frequencies in individuals with severe diabetic retinopathy (n=413), defined as incident retinal photocoagulation treatment, with individuals in whom little to no retinopathy had been observed at or after the baseline visit, defined as an ETDRS-score of <30 (n=630). Follow-up started at baseline, and ended in death, the occurrence of laser treatment, or at the end of available register data (Dec 31st 2015). After the onset of ESRD, follow-up was censored. Data on bacterial infections were collected from the Finnish National Drug Prescription Register, data on diabetic

retinopathy was collected from fundus photographs assessed by an ophthalmologist, and laser surgery data collected from the Finnish Hospital Discharge Register.

Study IV aim, cohort and phenotype

In study IV, we used GWAS to investigate whether there were common genetic variants associated with antibiotic purchase frequencies in individuals with diabetes. In GWAS, associations between common variations in the genome (SNPs) and different phenotypes can be investigated[273]. Using GWAS, it is possible to detect statistically significant differences in these SNPs across the whole genome between certain groups of interest, and hence, genetic variance associated with different clinical conditions or diseases can be found[274].

To increase the statistical power in the analysis, two separate GWAS-analyses were performed in the FinnDiane (n=5,092) and the DIREVA (n=4,247) participants respectively, after which the results were combined in a fixed effects meta-analysis. Replication analyses were performed in a Swedish cohort consisting of individuals with diabetes (All New Diabetics in Scania, ANDIS, n=9,602) and in a Finnish non-diabetic cohort with adults over 35 years old (FinnGen, n=159,666). In all Finnish cohorts, data on bacterial infections were collected from the Finnish National Drug Prescription Register.

Table 8. Summary of each study's design and methodologies

Parameter	Study I	Study II	Study III	Study IV
Study design	Register-based prospective follow-up study	Register-based prospective follow-up study	Register-based prospective follow-up study	GWAS
n	FinnDiane: 4,748 (4,169*); NDC: 12,954	3,781*	1,043*	FinnDiane: 5,092; DIREVA: 4,247, ANDIS: 9,602, FinnGen: 159,666
Phenotype of interest/main outcome	Infection frequency in T1D overall and different stages of DKD	Incident CHD: Death or hospitalization due to myocardial infarction, treatment with CABG or PCI	Incident SDR: Photocoagulation treatment of SDR	SNPs associated with infection susceptibility
Main measurement	Antibiotic purchases and bacterial infections treated within hospitals	Antibiotic purchases, LPS-activity	Antibiotic purchases, LPS-activity	Antibiotic purchases
Exclusion criteria	Unknown stage of DKD at baseline, death or emigration before 1996, special reimbursement of drug cost for diabetes mellitus 1996-2009	Prior history of CHD, unknown stage of DKD at baseline, progression of DKD during follow-up, ESRD	Prior laser treatment, unknown stage of DKD at baseline, ETDRS-score of 30-55.	Unavailable genetic data
Comorbidities adjusted for in the analyses	Age, sex, age at onset of diabetes, hypertension, CVD, atherosclerosis, cancers, mental disorders, neurological diseases, alcoholism, rheumatoid diseases, and chronic respiratory diseases	Age, sex, age at onset of diabetes, stage of DKD, history of smoking, systolic blood pressure, WHR, eGFR, non-HDL cholesterol and HbA_{1c}	Age, sex, duration of diabetes, history of smoking, systolic blood pressure, BMI, eGFR, LDL cholesterol and HbA_{1c}	Mean age during follow-up, sex, mean HbA_{1c} during follow-up, age at onset of diabetes, genotyping batch-components

*ANDIS indicates All New Diabetics in Scania; BMI, body mass index; CABG, coronary artery bypass graft surgery; CHD, coronary heart disease; CVD, cardiovascular disease; DIREVA, Diabetes Registry Vaasa; DKD, diabetic kidney disease; eGFR, estimated glomerular filtration rate; ETDRS, early treatment of diabetic retinopathy study; GWAS, Genome-wide association study; HbA_{1c}, glycated haemoglobin; LDL, low-density lipoprotein; LPS-activity, bacterial lipopolysaccharide activity; NDC, non-diabetic control; non-HDL, non-high-density lipoprotein; PCI, percutaneous coronary intervention; SDR, severe diabetic retinopathy; and WHR, waist-hip ratio. * Denotes number of participants with ascertained stage of diabetic nephropathy.*

4.5 Statistical analysis

Descriptive statistics

In all studies, the descriptive statistical results are expressed as mean (standard deviation [SD], for normal distributions) or median (interquartile range, IQR, for non-normal distributions), where appropriate. Statistical differences between two groups were assessed using the ANOVA test (normally distributed variables), Mann-Whitney U-test or the Kruskal-Wallis test (non-normally distributed variables) or Pearson's chi-square ($\chi 2$) test (categorical variables).

Detailed statistical analyses in Study I

To assess bacterial infections throughout the follow-up period, we calculated the cumulative number of both annual antibiotic purchases as well as bacterial infections treated within hospitals for all subjects from 1996 until death or the end of the year 2009. When the analyses were performed according to the nephropathy groups, the follow-up started at baseline. For subjects with no purchases or hospitalisations, we set the outcome to zero. The data was highly skewed with zeroes, due to which we applied zero-inflated Poisson regression models to compare infection frequencies between individuals with diabetes and NDCs, as well as differences between the different stages of nephropathy. As many individuals also had multiple events during the follow-up, we further implemented subject-specific random effects in the models. Results from the Poisson regression models on the differences in antibiotic purchases and hospitalisations due bacterial infections are reported as rate ratios (RRs).

Regarding comorbidities, using the National Drug Prescription register as well as the Care Register for Health Care, we identified diseases that may affect infection susceptibility for all individuals: cardiovascular disease, hypertension, atherosclerosis, cancer, mental disorders, neurological diseases, alcoholism, autoimmune diseases (rheumatoid arthritis), and chronic pulmonary disorders (asthma and obstructive pulmonary disease). These conditions were then used as covariates to adjust the rate ratios of the infections in the regression models.

When we compared individuals with diabetes with the NDCs, we included as covariates the age in 1996, sex, as well as the possible comorbidities identified from the national registers mentioned above. When we compared infection frequencies between the different stages of nephropathy, in addition to the covariates above, we further included duration of diabetes, sex, the calendar year, eGFR, smoking, and HbA_{1c} as covariates. The differences between groups were tested by using ANOVA, Mann-Whitney U test and the two-tailed Fisher's exact test, as applicable.

Detailed statistical analyses in Study II and III

Although study II and III differed in regard to complication outcome and phenotype, the studies were similar in regard to analysis approach. In both study II and study III, we investigated severe incident

diabetic complications (coronary heart disease and diabetic retinopathy, respectively), and applied survival regression models in order to provide an estimate of how bacterial infections could serve as risk factors for the complications. As exposure measurements, we used two covariates: the mean number of antibiotic purchases per follow-up year, as well as LPS-activity measured at baseline and reported as EU/ml. The mean number of antibiotic purchases was calculated for each subject as the total number of antibiotic purchases divided by the follow-up time in years. As individuals with incident diabetic complications had shorter follow-ups, compared to individuals without incident events, it resulted in a biased smaller number of total purchases in the individuals with incident complications. This bias was dealt with by the adjustment for the length of follow-up of the infection covariate while also resolving any time-dependency issues in the infection covariates in the proportional hazards regression models. Cumulative incidences of both incident coronary heart disease as well as severe diabetic retinopathy were calculated and estimated using Kaplan-Meier survival curves. In Cox regression models, we further adjusted the infection covariates for known risk factors of micro- and macrovascular disease: age, sex, age at onset of diabetes (study II) or duration of diabetes (study III), obesity (study II: WHR, study III: BMI), eGFR, stage of diabetic kidney disease (study II), dyslipidaemia (study II: non-HDL, study III: LDL), history of smoking and HbA_{1c}. In each study, several Cox regression models with an increasing number of covariates were built for both phenotypes, where the final models included all covariates.

In addition to using single baseline measurements of these risk factors as covariates in the models, we also implemented time-dependent Cox regression models, which allowed us to include multiple, prospective measurements of each risk factor so as to maximise the adjustment of the infection covariates, and enabled us to report the associations as accurately as possible. This is possible by building a function, which calls each prospective measurement and the corresponding time of the measurement for each risk factor into the model, and calculates a time-weighted average hazard ratio for the covariate using a stepwise function (**Fig 6)**, as demonstrated previously[275]. Using this approach in study II, we used all available longitudinal measurements of the traditional risk factors of coronary heart disease, while in study III, we used prospective ETDRS-scores to adjust for background retinopathy, to serve as a sensitivity analysis.

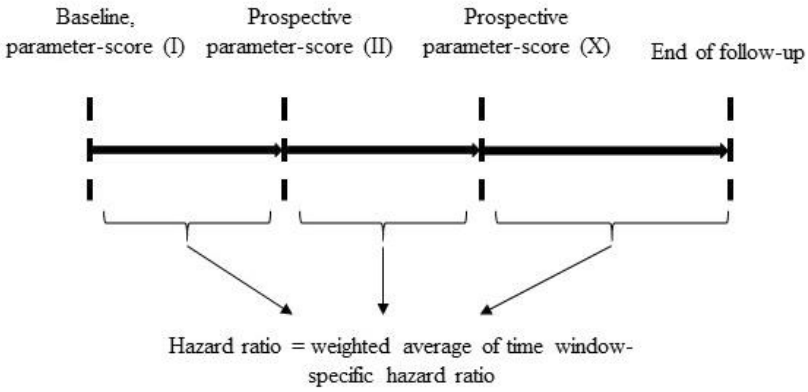


Figure 6. *Schematic figure of how the stepwise function calculates the time-weighted hazard ratio of multiple prospective measurements in a time-dependent Cox regression model.*

Detailed statistical analyses in Study IV

To assess whether any common genetic mutations associate with infection susceptibility in diabetes, we calculated an infection frequency risk score for all subjects included in the GWAS. Due to an excess of zeroes in the data, we added a small constant to the data before a logarithmic transformation: $\log_e$([total number of purchases during follow-up/follow-up time in years] + 0.5 × minimum non-zero value). We calculated the mean HbA_{1c} each subject had during the follow-up and added the glycaemic controls as a covariate into the analysis to adjust for glycaemic control as an environmental factor increasing the risk of infections. Furthermore, the analysis was adjusted for sex, the mean age during follow-up, age at onset of diabetes, and genotyping batch-specific components. To estimate the narrow-sense heritability, i.e., the proportion of the variance in the phenotype attributable to variance in the genetic variants, we used a genetic relationship matrix of unrelated individuals in a mixed linear model. In addition to the GWAS, a pathway analysis was performed to search for biologic pathways enriched for our infection susceptibility risk score.

In the GWAS analyses, quality control filters were assigned as a minor allele frequency (MAF) ≥ 0.01 and imputation information (r2) of ≥ 0.7). After genotyping and imputation, the FinnDiane cohort had 8.4×10^6 and the DIREVA cohort had 8.6×10^6 SNPs available for the GWAS. The GWAS analyses in the FinnDiane and DIREVA cohorts were performed with estimated allele dosages and a linear mixed model.

4.6 Software

Analyses in study I were performed using SAS 9.2. Analyses in study II and III were performed using the openly available R-software versions 3.5.2-4.0.2. Time-dependent Cox analyses in study II and III were performed using the *tmerge* function in the *survival*-package in R-software.

In study IV, GWAS and meta-analysis was performed using RVTESTS software. DNA samples were genotyped using the HumanCoreExome BeadChips-12 v. 1.0, -12 v. 1.1, or -24 v. 1.0 BeadChip (Illumina, San Diego, CA) in both the FinnDiane and the DIREVA cohorts. Genotypes were imputed with the minimac 3 software and the 1000 Genomes reference panel. Heritability estimates were investigated with the Genome-wide Complex Trait Analysis (GCTA) software[276]. Genome-wide pathway analysis was performed with the Pascal (Pathway Scoring Algorithm) -software[277].

4.7 Clinical measurements

Diabetic Kidney Disease

The severity of diabetic kidney disease was assessed by the AER in at least two of three overnight or 24 h urine collections: normal AER (<20 µg/min or <30 mg/24 h), microalbuminuria (≥20 <200 µg/min or ≥30 <300 mg/24 h), macroalbuminuria (≥200 µg/min or ≥300 µg/24 h), and ESRD (defined as dialysis treatment or kidney transplantation). During fever or menstruation, urine samples were not collected.

Bacterial lipopolysaccharides

Serum LPS-activity levels were measured with the LAL chromogenic end-point assay (Hycult)[181]. All LPS-measurements were performed on serum samples collected at baseline. In previous analyses in FinnDiane, we have observed a correlation between LPS-concentrations and the time the sample has spent frozen (unpublished data), due to which the freezing time of the sample was included as a covariate in the analyses when necessary.

Blood pressure

The blood pressure was measured at all FinnDiane study visits using either a standardized automatic blood pressure device, or a manual mercury sphygmomanometer. The measures were performed twice, in the sitting position, by a nurse after a rest of 10 minutes, and the mean value of the two measurements were calculated, for the systolic and diastolic pressure, separately.

Grading of diabetic retinopathy

In study III, the stage of diabetic retinopathy was assessed according to the ETDRS-scale (**Table 2)**, and individuals were categorized into groups based on the ETDRS-score as no/mild retinopathy

(ETDRS-score of < 30 at or after baseline) or mild to very severe retinopathy (ETDRS-score of ≥ 30 to 53 at baseline).

Kidney function

The eGFR was calculated for all individuals based on the creatinine measurements from serum samples using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula[278].

History of smoking

Smoking was used in studies I-III as a dichotomous variable, positive if the subject had ever smoked or was currently smoking.

HbA_{1c} and lipid data

All HbA_{1c} and lipid data were measured from blood samples, collected at study visits and analysed using established methods. LDL-concentrations were calculated with the Friedewald equation.

4.8 Ethical aspects

The study protocol is in accordance with the declaration of Helsinki, and it has been approved by the local ethics committees. All participants gave written consent prior to the participation in the study and the participants gained no immediate benefits by participating. They could cancel their participation at any point, without providing a reason. All participants were pseudo-anonymous through coded study-IDs and all individual data are stored in a locked compartment with access only to a few selected researchers for 20 years. The study designs in study I-IV are observational, and as there are no interventions made, there are no immediate ethical concerns regarding the design. All potential conflicts of interest are reported in each study. The supporters of our studies are not involved in any part of the analysis or publication process (including study design, data collection, analysis/interpretation of the results, or preparation of any manuscripts). Potential damage to the participants is minimal and mainly restricted to discomfort caused by blood sampling (e.g., blood samples) as well as the time loss they spend during study visits.

5. RESULTS

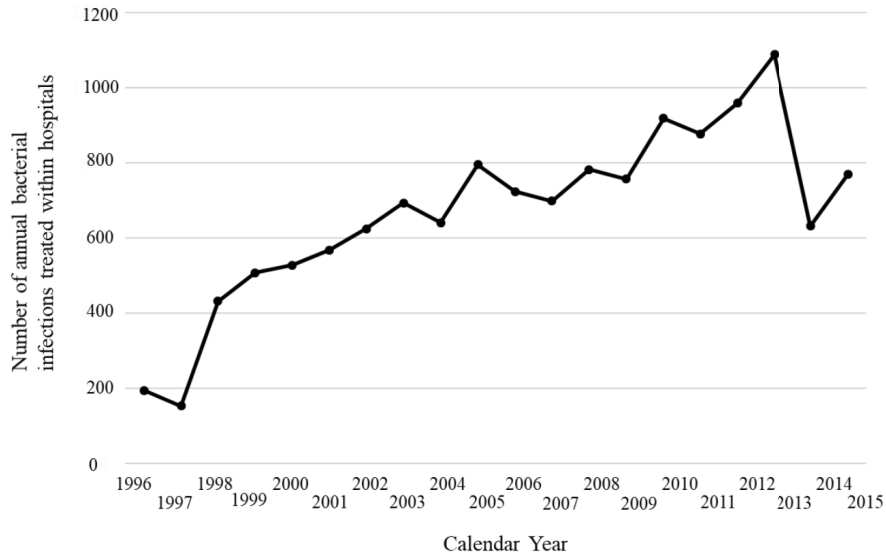
This chapter presents the results from studies I-IV. As a short summary of the study aims, in study I, we surveyed the frequency of bacterial infections treated both outside and within hospitals in individuals with type 1 diabetes by investigating the frequency of antibiotic purchases and hospitalisations due to bacterial infections. We compared these infection rates to the corresponding rates in age- and sex-matched NDCs and further studied how the infection frequencies associated with different stages of diabetic kidney disease as well as glycaemic control. In studies II and III, we investigated if antibiotic purchases and endotoxemia were associated with severe incident chronic complications of diabetes and whether they served as independent risk factors for the development of the complications. Finally, in study IV, we studied potential genetic risk factors for the susceptibility to bacterial infections in individuals with diabetes using a GWAS approach.

5.1. Annual infection frequencies in type 1 diabetes between 1996-2015 (unpublished observations, JR Simonsen et al.).

In the whole FinnDiane cohort, between Jan 1st 1996 and Dec 31st 2015, there were 146,928 antibiotic prescription purchases in total, and an approximately ten-times lower number of bacterial infections treated within hospitals (n=13,348). We observed a rising number of bacterial infections treated in hospitals in the Hospital Discharge Register, up until 2014, when the number dropped from over 1,000 inpatient treated infections to a bit over 600 infections, from which the number of infections again increased in 2015 (**Fig 7A**). Although the reasons for the increasing number of bacterial infections observed between the years 1997 to 2014 are obscure, one reason might be the progressing duration of diabetes and the increasing numbers of diabetic complications, including ESRD, a critical risk factor for bacterial infections, especially septicaemia. The reason for the sudden drop in infections in 2014 is unknown.

Compared to the bacterial infections treated in hospitals, the total numbers of antibiotic prescription purchases were far greater, ranging between 6000-8000 purchases annually and displayed a more stable trend during the 20-year time period (**Fig 7 B**).

A.



B.

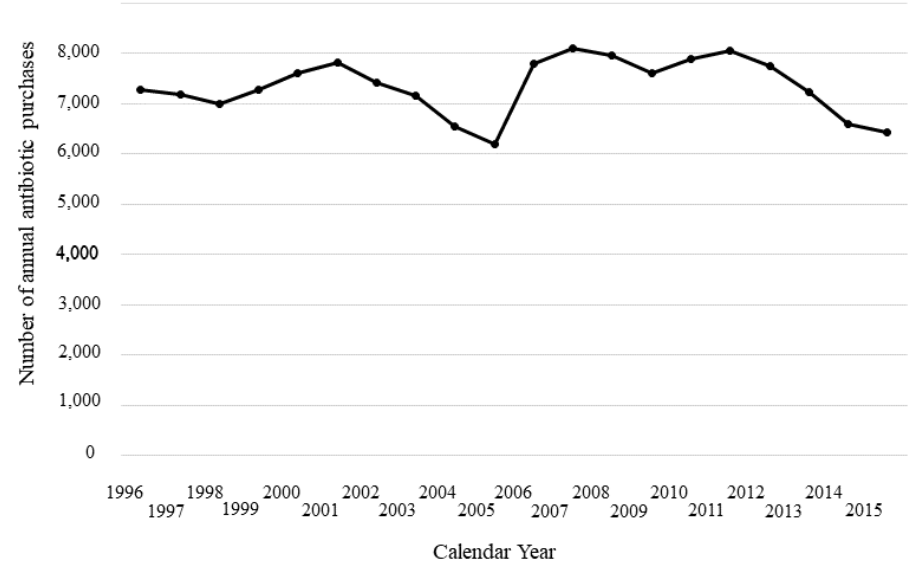


Figure 7. *The total annual number of a) bacterial infections treated within hospitals, and b) antibiotic prescription purchases, between 1996 to 2015 in FinnDiane subjects.*

5.2 Antibiotic purchase profiles in type 1 diabetes between 1995-2015 (unpublished observations, JR Simonsen et al.).

For this thesis, as a post-hoc analysis, we further assessed more closely what kinds of antibiotics had been purchased by the FinnDiane subjects during the years 1996 to 2015, by using the ATC-codes recorded in the Finnish National Drug Prescription Register. We found that roughly 55% of all antibiotics were either cephalosporins or penicillin-based antibiotics (**Table 9**) in the whole FinnDiane cohort, while tetracyclines and fluoroquinolones constituted 10% of the purchases each. Sulphonamides and trimethoprim, which can be considered in general to predominantly reflect the treatment of urinary tract infections, constituted about 6% of the total number of purchases.

Table 9. The antibiotic purchase profiles of all FinnDiane subjects between Jan 1st 1996 to Dec 31st 2015.

Antibiotic according to ATC-group	Number of antibiotic purchases (% of all purchases)
J01A Tetracyclines	16,584 (10.8)
J01B Amphenicols	0 (0)
J01C Beta-lactam antibacterials, penicillins.	42,369 (27.4)
J01D Other beta-lactam antibacterials	43,294 (28.0)
J01E Sulphonamides and trimethoprim	9,335 (6.1)
J01F Macrolides, lincosamides and streptogramins.	25,537 (16.6)
J01G Aminoglycoside antibacterials	37 (<0.1)
J01M Quinolone antibacterials	15,865 (10.3)
J01R Combinations of antibacterials	0 (0%)
J01X Other Antibacterials	1,201 (0.8)
Total	154,222 (100%)

Data is presented as n (% of all antibiotic purchases). ATC indicates Anatomical Therapeutic Chemical Classification.

5.3 Bacterial infection frequencies in individuals with type 1 diabetes vs. the general population

In study I, we found that, compared to NDCs matched for sex and age, individuals with type 1 diabetes had significantly more frequent bacterial infections treated in hospitals as well as antibiotic purchases. The annual purchase rate of antibiotics was 1.00 (95% CI: 1.00 to 1.01) and the annual hospitalisation rate due to bacterial infections/1000 follow-up years was 62.0 (60.1 to 64.0) for individuals with type 1 diabetes, while the corresponding numbers in the NDCs were 0.47 (0.46 to 0.47) and 16.3 (15.7 to 16.9), respectively. When comparing infection rates in zero-inflated Poisson regression models adjusted for comorbidities, we found that individuals with type 1 diabetes had a roughly two-fold higher risk of contracting a bacterial infection, identified as either an oral antibiotic treatment or hospitalisation compared to the controls (rate ratio [RR]: 1.71 [95% CI: 1.65-1.77]; and 2.30 [2.11-2.51], respectively). To further minimize the effect of comorbidities on the differences observed in the infection frequencies, we also compared antibiotic purchase frequencies between type 1 diabetes individuals with a sustained normal albumin excretion rate with their age- and sex-matched control subjects. In this comparison, individuals with type 1 diabetes purchased roughly 1.5-times more antibiotics compared to the NDCs, after adjustment for comorbidities (RR 1.48 [1.41-1.55].

We further compared the incidence of different specific bacterial infection foci between individuals with type 1 diabetes and the NDCs, according to the infection foci grouping presented in **Table 7**. The greatest observed differences between the two groups were in the frequencies of sepsis (4-fold greater in type 1 diabetes [308 recorded cases vs 76, $P<0.05$]), osteomyelitis (6.4-fold greater in type 1 diabetes [210 cases vs 33, $P<0.05$]), peritonitis (41-fold greater in type 1 diabetes [327 cases vs 8, $P<0.05$]). Of note, these analyses included all individuals with type 1 diabetes, and no sub-stratifications or adjustments for comorbidities were performed. Therefore, the presence of diabetic complications explain, at least in part, the greater frequencies observed in the individuals with type 1 diabetes: ESRD and peritoneal dialysis greatly increase the risk for peritonitis and/or sepsis, while peripheral neuropathy in conjunction with peripheral artery disease may cause chronic ulcers, elevating the risk of osteomyelitis.

5.4 Bacterial infections and diabetic kidney disease

In order to assess the relationship between bacterial infections and the different stages of diabetic kidney disease, we calculated antibiotic purchase rates as well as the rates of bacterial infections treated within hospitals, for the follow-up years of each stage of diabetic kidney disease. Antibiotic purchase rates were calculated as the mean number of antibiotic purchases per follow-up year, while the hospitalisation rates were calculated as the mean number of hospitalisations per 1,000 years, as the rates of bacterial infections treated within hospitals were markedly lower compared to antibiotic purchases. During 1998-2009, the total number of follow-up years for each stage of diabetic kidney disease was as follows: 19,804 follow-up years for normal AER; 4,958 for microalbuminuria; 5,821 for macroalbuminuria; 856

for dialysis and finally; 2,250 for kidney transplant. When comparing infection frequencies across these groups, we found that the mean number of antibiotic purchases per follow-up year associated significantly with the stage of diabetic kidney disease (**Fig 8**): during normal AER, individuals bought, in average, 0.65 antibiotic purchases per year (95% CI: 0.64-0.66); during microalbuminuria, 0.94 (0.91-0.96); during macroalbuminuria, 1.18 (1.15-1.21); during dialysis, 3.23 (3.11-3.35); and finally, after receiving a kidney transplant 3.03 antibiotics per year (2.95 to 3.10). All differences between the groups were significant, as was the trend of increasing purchases from normal AER to dialysis (P <0.0001 for trend, Kruskal-Wallis test). Similarly, as in the data with antibiotic purchases, we observed a trend of increasing rates of bacterial infections treated within hospitals as the severity of diabetic kidney disease increased (**Fig 9**).

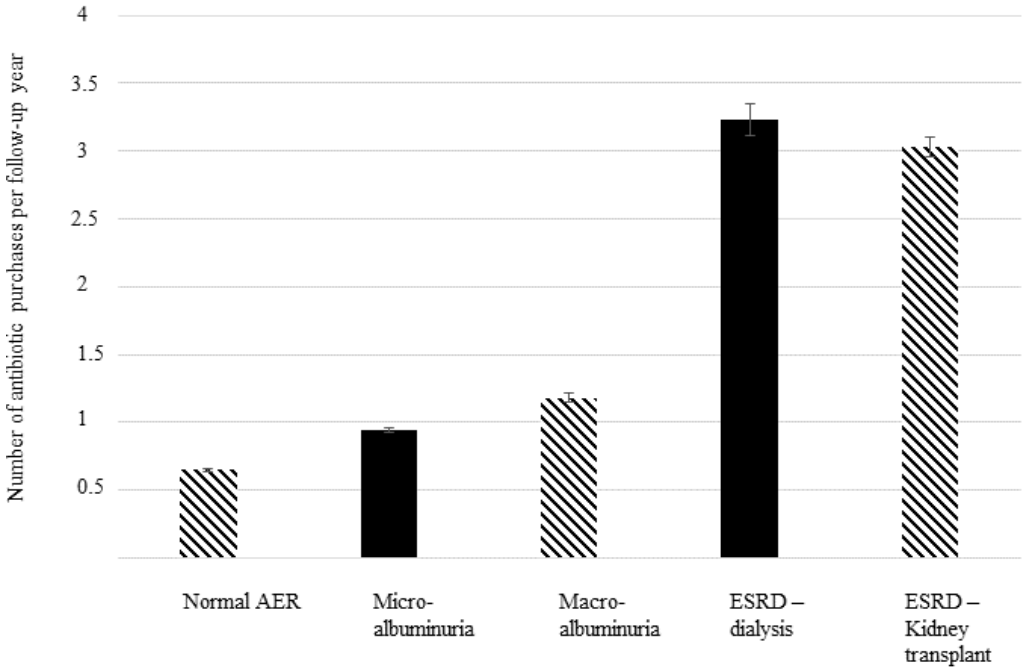
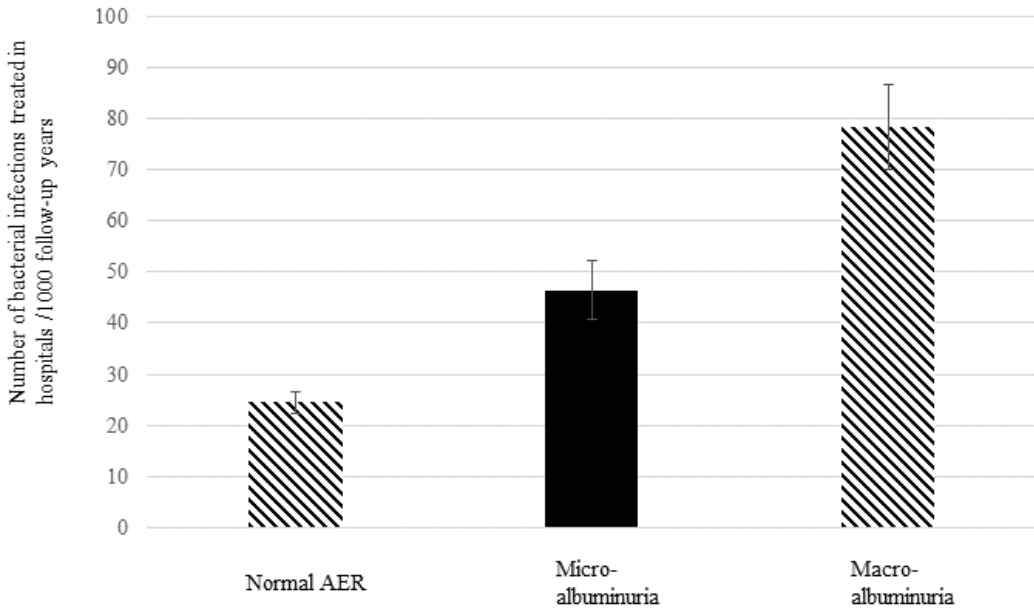


Figure 8. *The mean number of antibiotic purchases per follow-up year with 95% confidence intervals in FinnDiane subjects in each stage of diabetic kidney disease between 1998-2009. AER indicates albumin excretion rate; ESRD, end-stage renal disease.*

A.



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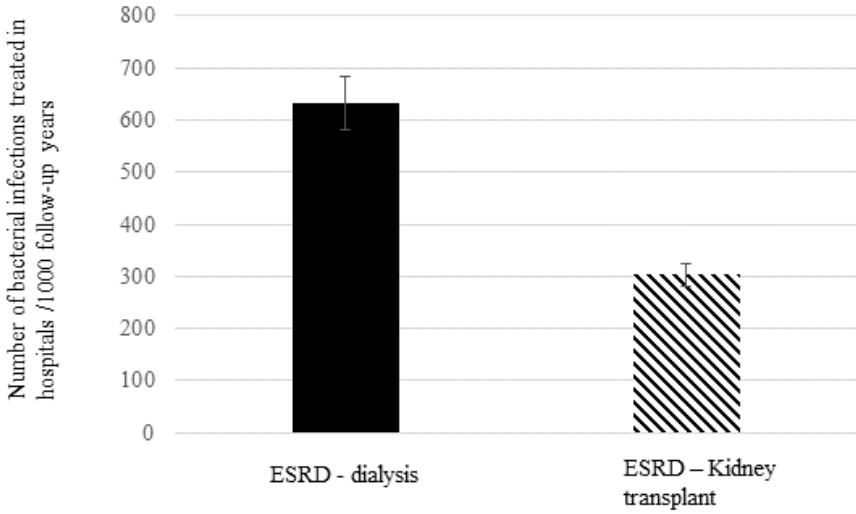


Figure 9. *The mean number of bacterial infections treated in hospitals per 1,000 follow-up years with 95% confidence intervals in FinnDiane subjects in A) normal AER, micro- and macroalbuminuria, and B) dialysis and kidney transplant, between 1998-2009. AER indicates albumin excretion rate; and ESRD, end-stage renal disease.*

We further compared infection rate ratios in the different stages of diabetic kidney disease to each other by applying fully adjusted zero-inflated regression models with the mean number of antibiotic purchases per follow-up year as the main covariate, and compared the purchase frequencies in normal AER to microalbuminuria, macroalbuminuria and finally ESRD. We found that microalbuminuria increased antibiotic purchase frequencies 1.2-fold (95% CI: 1.1-1.3); macroalbuminuria 1.3-fold (1.2-1.4); dialysis, 2.4-fold (2.1-2.8); and kidney transplant, 2.7-fold (2.4-3.2), compared to the individuals with a normal AER.

Bacterial infections and incident microalbuminuria

To investigate the connection between bacterial infections and the development of albuminuria, we identified individuals with a normal AER at baseline, but who developed microalbuminuria during follow-up (n=219). For these individuals, we selected control individuals with type 1 diabetes (n=874), who retained a normal AER throughout the follow-up and that were matched for age, sex, and diabetes duration (±2 years) to the individuals with incident microalbuminuria. The annual number of total antibiotic purchases per subject was calculated for each year, up to four years before, during, and three years after the onset of microalbuminuria for both groups. For the control individuals, the antibiotic purchases were calculated for the same calendar years as the assigned individuals with incident microalbuminuria. In these analyses, we observed a significant increase in the number of annual antibiotic purchases three years before, the year before, during, and after the onset of microalbuminuria (**Fig 10**). We also saw an increase in antibiotic purchase frequencies in the individuals with incident microalbuminuria during the year of the onset of microalbuminuria, compared to four years before. The results were adjusted for sex, HbA_{1c}, and the duration of diabetes.

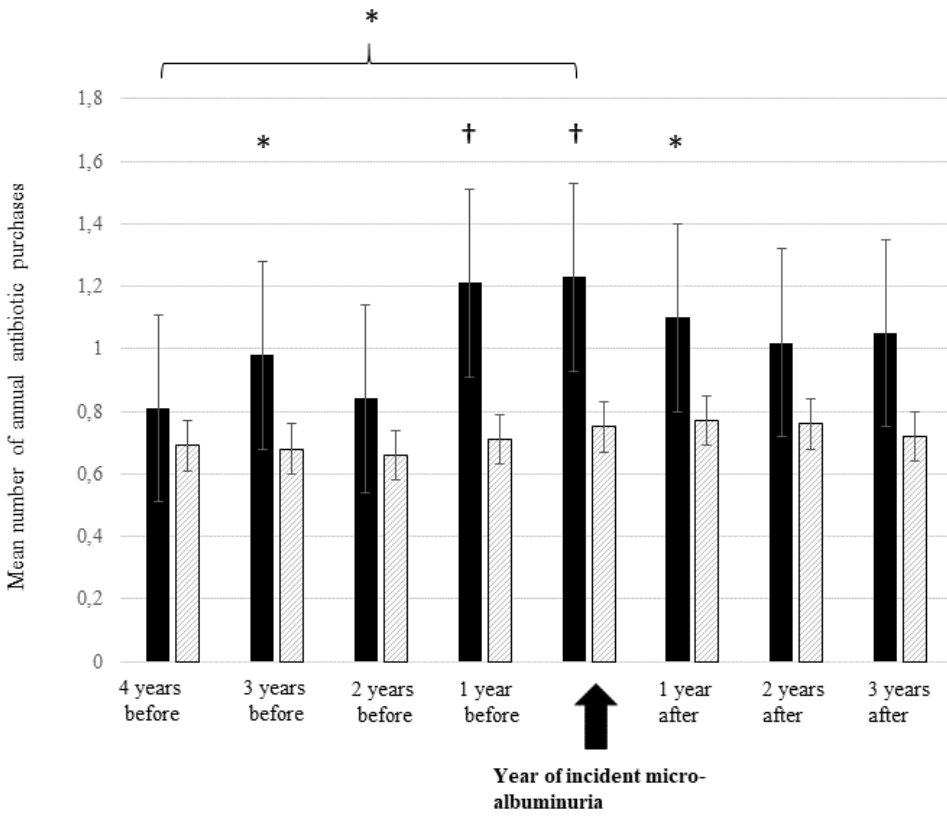


Figure 10. *The mean annual number of antibiotic purchases (with 95% confidence intervals) between individuals with type 1 diabetes and incident microalbuminuria (black columns), and controls with type 1 diabetes with a sustained normal AER (striped columns), adjusted for age, sex, and diabetes duration (±2 years). The top bracket indicates the difference between the annual number of purchases four years before versus during the same year as incident microalbuminuria in the group with incident microalbuminuria. * indicates P < 0.05, and † indicates P < 0.01.*

5.5 Bacterial infection frequencies and hyperglycaemia in type 1 diabetes

As previous studies have suggested that hyperglycaemia is an important risk factor for infections, we investigated how the infection frequency associated to different levels of chronic hyperglycaemia in individuals with type 1 diabetes. This was performed using two separate approaches.

Firstly, HbA_{1c} was added into the zero-inflated regression models as a continuous covariate and adjusted for comorbidities. These analyses were performed separately, according to the stage of diabetic kidney disease at baseline. In these models, each percentage point increase in baseline HbA_{1c} was associated with a 6% (Odds Ratio [OR]: 1.06 [95%CI: 1.03-1.09]) increase in antibiotic purchases in individuals with normal AER, a 10% (OR: 1.10 [1.04-1.16]) increase in the purchases in individuals with

microalbuminuria and a 7% (OR: 1.07 [1.02-1.13]) increase in the purchases in individuals with macroalbuminuria. In individuals with ESRD, the findings were non-significant. The corresponding numbers when the outcome was a bacterial infection treated within a hospital were greater: 26% (OR: 1.26 [1.15-1.37]) for individuals with normal AER, 15% (OR 1.15 [1.01-1.31]) for individuals with microalbuminuria and finally, 28% (OR 1.28 [1.16-1.41]) for individuals with macroalbuminuria.

Secondly, we stratified individuals into five different groups, according to their baseline HbA_{1c}: <7%, 7-7.9%, 8-8.9%, 9-9.9% and ≥10%, and calculated the total number of purchases made within a three-year period: the year before, during, and after the baseline visit. These analyses were performed separately for individuals with a normal AER at baseline, as well as for individuals with micro- or macroalbuminuria pooled into one group. Here, individuals with a normal AER and an HbA_{1c} ranging between 7-7.9% at baseline had 1.2-fold higher antibiotic purchase frequencies (95% CI: 1.1-1.3]), and individuals with an HbA_{1c} ranging between 8-10% had 1.5-fold (1.4-1.6) as many antibiotic purchases, compared to individuals with a normal AER and a glycaemic control within the optimal range (<7.0%). In the individuals with micro- or microalbuminuria the differences in rate ratios were smaller between individuals with poor glucose control when compared with individuals with optimal glucose control. Significant differences between these groups were only observed when we compared individuals with a baseline HbA_{1c} exceeding 9% to individuals with optimal glycaemic control (RR 1.2 [1.1-1.4]).

5.6 Antibiotic purchases as risk factors for severe diabetic complications – coronary heart disease and diabetic retinopathy

In study II and III, to evaluate the association between the development of severe chronic complications of diabetes and bacterial infections, we calculated the mean number of antibiotic purchases per follow-up year for all individuals included in the studies and used it as a measure of bacterial infection frequency. We then applied survival regression analysis to investigate the association between the antibiotic purchases and incident severe events of coronary heart disease (study II) and diabetic retinopathy (study III). Between 1996 and 2015, we identified 370 individuals with an incident coronary heart disease event and 413 individuals with incident severe diabetic retinopathy. As controls for study II and III, 3,411 and 630 individuals, respectively, were found eligible, according to the selection criteria described in chapter 4.4.

In study II, we identified in total 43,313 antibiotic purchases during the follow-up. Individuals with incident coronary heart disease had a 1.6-fold greater mean number of antibiotic purchases per follow-up year: 1.3 (standard deviation ± 1.8), compared to a mean purchase frequency of 0.8 (± 0.8) in the individuals without incident coronary heart disease (P<0.001). In study III, due to the smaller number of participants and shorter follow-up periods the total number of purchases was also lower, and 9,214 antibiotic purchases were identified during the follow-up. Individuals with incident severe diabetic retinopathy demonstrated a 1.4-fold higher frequency of annual antibiotic purchases compared to those

without diabetic retinopathy: 0.92 (± 1.04) antibiotic purchases per year vs 0.67 (± 0.68), P=0.02, respectively.

Antibiotic purchase frequencies and cumulative hazard of severe diabetic complications

In both study II and III, we stratified the cohorts according to the antibiotic purchase frequencies, into individuals with frequent purchases (at least one antibiotic on average annually), or infrequent purchases (less than one purchase on average annually), and calculated the cumulative hazard of the diabetic complication outcomes. In both studies, we observed a significant difference in the incidence of severe chronic complications, between individuals with frequent antibiotic purchases and those with infrequent purchases (**Fig 11**). In study II, individuals with frequent purchases (n=1,045) had a 1.8-fold greater cumulative incidence of coronary heart disease (14.5% vs 8.0%, χ2-test: P<0.0001), compared to individuals with infrequent purchases (n=2,736) (**Fig 11A**). When the outcome was severe diabetic retinopathy, individuals with frequent purchases (n=269) had a 1.5-fold greater cumulative incidence of severe diabetic retinopathy (52% vs 35%, P<0.001), compared to those with infrequent purchases (n=774) (**Fig 11B**).

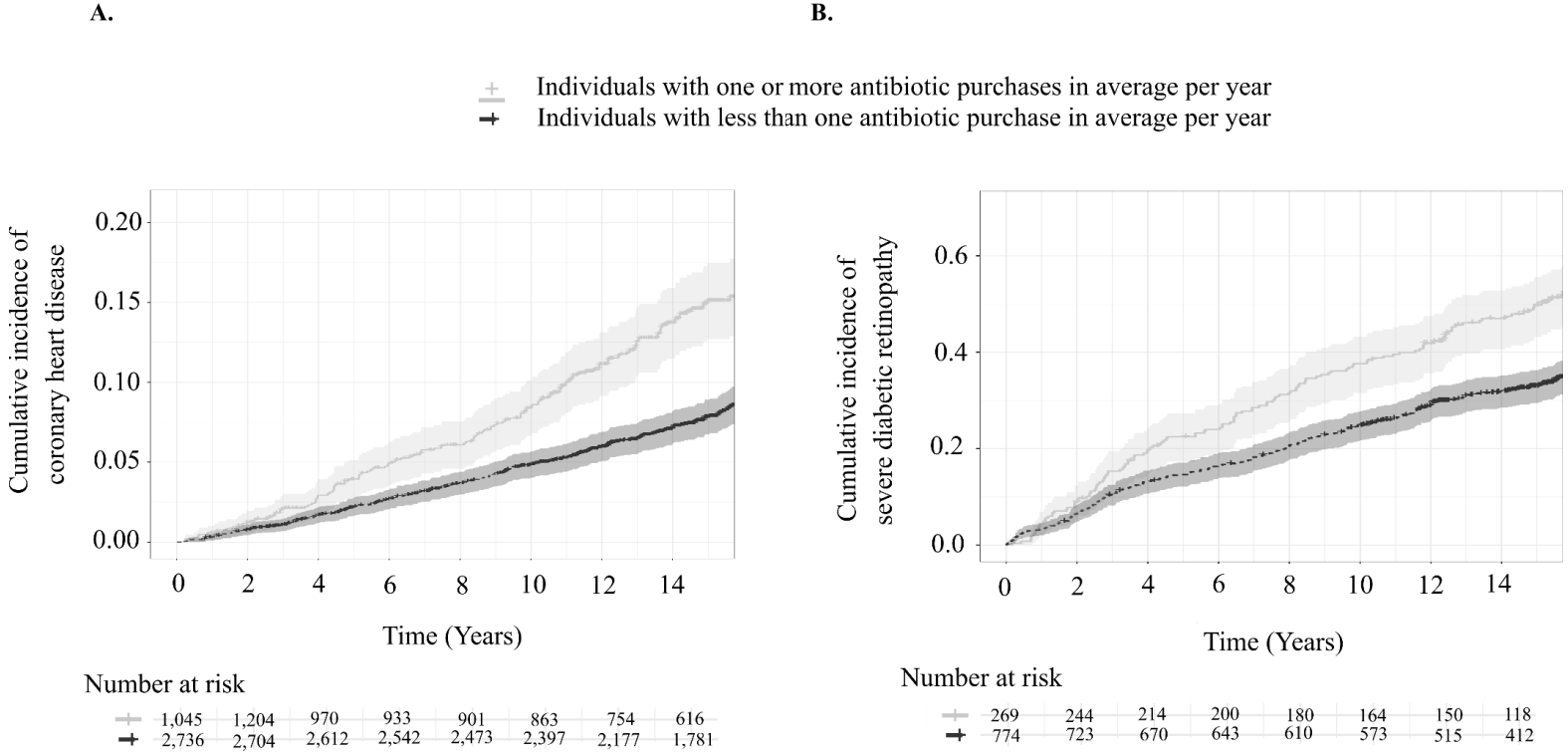


Figure 11. *Kaplan–Meier cumulative incidence curves for A) coronary heart disease, and B) severe diabetic retinopathy, over a follow-up of 15 years in individuals with type 1 diabetes and frequent antibiotic purchases (at least one antibiotic purchase in average annually) versus individuals with infrequent purchases (less than one purchase in average annually).*

Antibiotic purchase frequency as an independent risk factor of severe diabetic complications

By applying Cox proportional hazards regression models, we assessed the mean number of antibiotic purchases per follow-up year as a risk factor for incident coronary heart disease and severe retinopathy. We adjusted the models for traditional risk factors of the complications in three separate models, where the first model contained the unadjusted hazard ratio of the antibiotic purchases, the second model further including static confounders (age, sex, age at onset of diabetes [study II] or duration of diabetes [study III], and stage of diabetic kidney disease at baseline [study II]), and the final model further including all dynamic confounders (systolic blood pressure, eGFR, non-high-density lipoprotein cholesterol concentrations [study II] or low-density lipoprotein cholesterol [study III], waist-hip ratio [study II] or body mass index [study III], HbA_{1c}, and finally, history of smoking).

In these proportional hazards regression models, the mean number of antibiotic purchases per follow-up year was a significant risk factor for coronary heart disease, even after adjustment for all confounders (**Table 10A**). In the third and fully adjusted model, each unit increase in the annual purchase rate was associated with a 20% increase in the risk of incident coronary heart disease (HR: 1.21, 95% CI: 1.14-1.29, P<0.0001). In study II, we further used all available longitudinal measurements on dynamic confounders acquired during prospective study visits to maximize the accuracy of the adjustment of the Cox regression model. The longitudinal data were introduced as time-dependent covariates into the models, and time-weighted average hazard ratios from the specific time-windows between the measurements were calculated. Interestingly, this major adjustment had little impact on the antibiotic purchases as risk factors for incident coronary heart disease (HR: 1.20, 95% CI: 1.13–1.20, P<0.001).

Regarding severe diabetic retinopathy (**Table 10B**), the antibiotic purchase rate was a significant risk factor after the adjustment of static confounders (HR: 1.16 [1.05-1.27], P=0.002), however, introducing dynamic covariates further into the model resulted in a non-significant association (HR: 1.09 [0.98-1.21], P=0.11). As the individuals had varying degrees of diabetic retinopathy at baseline, we used prospective measurements of ETDRS-scores in a time-dependent Cox regression model, to adjust for the degree of background retinopathy for all subjects. Thus, we assessed the annual antibiotic purchase rate as a risk factor for severe diabetic retinopathy, while assuming the level of background retinopathy both at baseline as well as during the follow-up to be equal across the subjects included in the analysis. In these models, while further adjusting for age, sex, and the duration of diabetes, the antibiotic purchase rates were non-significant risk factors for severe diabetic retinopathy (HR: 1.05 [0.93–1.18], p=0.43).

Table 10. Results from the multivariable Cox proportional hazards regression models, demonstrating the mean number of antibiotic purchases per follow-up year as a risk factor for A) incident coronary heart disease, and B) incident severe diabetic retinopathy, in FinnDiane subjects with type 1 diabetes.

A.

Confounders included	HR (95% CI) for the mean number of UTI-antibiotic purchases per follow-up year	P
Model 1: NA	1.42 (1.35-1.50)	<0.0001
Model 2: Age, sex, age at onset of diabetes, stage of DKD at baseline	1.22 (1.15-1.29)	<0.0001
Model 3: Age, sex, age at onset of diabetes, non-HDL, eGFR, HbA_{1c}, WHR, systolic blood pressure, stage of DKD at baseline, history of smoking	1.21 (1.14-1.29)	<0.0001

B.

Confounders included	HR (95% CI) for the mean number of UTI-antibiotic purchases per follow-up year	P
Model 1: NA	1.29 (1.18-1.42)	<0.0001
Model 2: Age, sex, duration of diabetes	1.16 (1.05-1.27)	0.002
Model 3: Age, sex, duration of diabetes, LDL, eGFR, HbA_{1c}, BMI, systolic blood pressure, history of smoking	1.09 (0.98-1.21)	0.11

NA indicates not applicable; UTI, urinary tract infection; HR, hazard ratio; HbA_{1c}, glycated haemoglobin; non-HDL, non-high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; WHR, waist-hip ratio; DKD, diabetic kidney disease; BMI, body-mass index.

Antibiotics used in the treatment of urinary tract infections as risk factors for incident coronary heart disease ***(unpublished observations, JR Simonsen et al.)***.

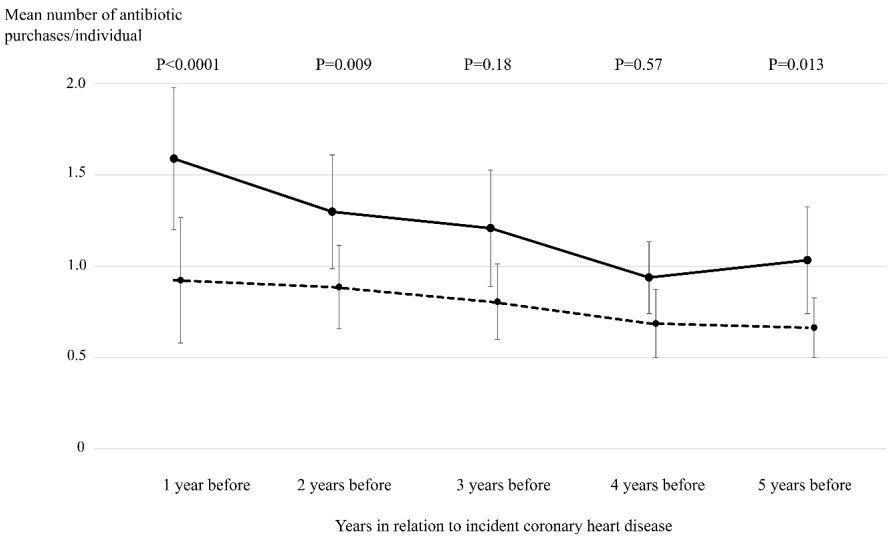
As a post-hoc analysis of study II, we included only antibiotics mainly used in the treatment of urinary tract infections (**Table 6B**) and identified, in total, 8,330 urinary tract antibiotic purchases (19.2% of all antibiotic purchases), by 1,687 individuals in total, during the whole follow-up period. The remaining individuals of the cohort (n=2,086) purchased none of these antibiotics. We further counted the mean number of urinary tract infection antibiotic purchases per follow-up year and applied Cox proportional hazards regression models as previously (**Table 10A**). Interestingly, these urinary tract antibiotic

purchases were also independent risk factors for incident coronary heart disease, even in fully adjusted Cox proportional hazards regression models (identical in terms of adjustments as to **Table 10 A**, model 3) with a hazard ratio of 1.31 (95% CI: 1.28 to 1.35), P<0.001. Conversely, excluding these urinary tract antibiotics from the analysis and including all other antibiotic purchases (n=34,983, 80.8% of all antibiotic purchases) into the analyses demonstrated a nearly identical hazard ratio and an equal level of significance: 1.30 (95%CI: 1.19 to 1.42, P<0.001). Due to the lower number of subjects and antibiotic purchases in study III, this post-hoc analysis was performed solely in study II.

Annual antibiotic purchase rates before incident severe diabetic complications

To more closely assess the antibiotic purchase rates before incident coronary heart disease and incident severe diabetic retinopathy in type 1 diabetes, we identified all individuals with an incident complication, to which we could assign a control individual with type 1 diabetes but without such complications, of the same age, sex, duration of diabetes (±3 years), and stage of diabetic kidney disease. For these case-control pairs, we calculated for each individual, the annual number of antibiotic purchases before the incident complications. With incident coronary heart disease as outcome, we were able to identify 211 cases for whom we found an eligible control-individual (n=422 in total), while we identified 158 eligible case-control pairs with incident severe diabetic retinopathy as the outcome (n=316 in total). In both analyses, we observed higher antibiotic purchase frequencies in the individuals developing incident complications, particularly one and two years before the complications, as compared to the matched controls (**Fig 12).** Importantly, the cases and controls were matched for the age and duration of diabetes at baseline, due to which no significant differences in age or duration of diabetes were observed at the calendar year level, and the antibiotic purchases in both groups were made during the same calendar years, addressing this potential methodological bias.

A.



B.

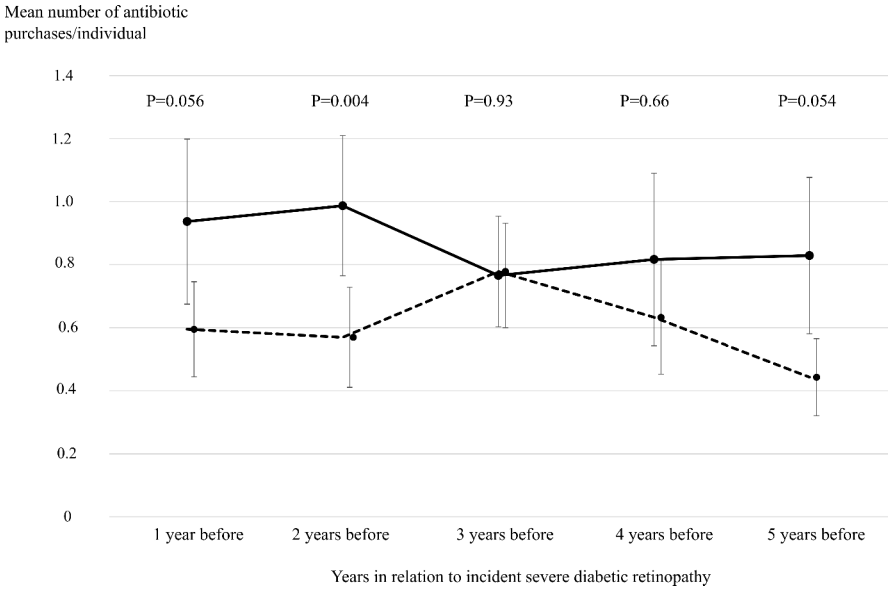


Figure 12. *The annual mean number of antibiotic purchases per individual with 95% confidence intervals in case-control pairs before A) incident coronary heart disease, and B) severe diabetic retinopathy. Controls were matched for age (within one year), sex, diabetes duration (+/- 3 years), and stage of diabetic nephropathy. Number of case-control pairs in A: 211 (422 individuals in total), and B: 158 (316 individuals in total). Cases are indicated by a continuous line, controls by a dotted line.*

5.7 Endotoxemia as a risk factor for severe diabetic complications - incident coronary heart disease and diabetic retinopathy

Using a similar methodologic approach as with the register data on antibiotic purchases, we assessed endotoxemia as a risk factor for coronary heart disease and severe diabetic retinopathy in the FinnDiane participants. In study II, LPS-activity was available for 3,342 individuals, while in study III, the measurements were available for 879 individuals.

Endotoxemia and cumulative hazard of severe diabetic complications

To study the effect of LPS-activity on the cumulative risk of developing coronary heart disease or diabetic retinopathy, we stratified individuals in both studies according to the interquartile range of LPS-activity: High LPS (LPS≥0.73), moderately high LPS (LPS≥0.51-<0.73), moderately low LPS (LPS≥0.36-0.51), and finally low LPS-activity (LPS<0.36 EU/ml). The interquartile range of LPS-activity was the same in both studies. In study II, we observed a markedly increased cumulative incidence of coronary heart disease in the individuals with high levels of LPS activity (cumulative incidence: 12.6% [95%CI: 10.3-14.8]) as compared to individuals with moderately low LPS (7.3% [5.6-9.1], χ2-test: P<0.001) as well as low LPS (9.0% [0.07-11.1], P=0.03) (**Fig 13A**). The differences in the incidence of coronary heart disease between individuals with high LPS compared with moderately high LPS (9.6% [7.6-11.6]), were borderline statistically significant (P=0.07). No significant differences in the cumulative hazard of incident coronary heart disease were observed between groups with moderately high, moderately low or low LPS. We found corresponding results in study III with severe diabetic retinopathy as outcome, where stratification according to the interquartile range of baseline endotoxemia, showed that the incidence of severe diabetic retinopathy was highest in the group with high LPS: 46.7% (95% CI: 40.1 to 53.2). This risk was significantly greater compared with all the other groups: low LPS activity (cumulative incidence: 36.5% [29.8-43.1], P=0.04), moderately low LPS activity (36.9% [30.7-43.2], P=0.04) and moderately high LPS (35.2% [28.8-41.6], P=0.02) (**Fig 13B**).

Endotoxemia as an independent risk factor of severe diabetic complications

In multivariable Cox proportional hazard models in study II, endotoxemia was found to be a significant risk factor for incident coronary heart disease (**Table 11A**), after adjusting for the freezing time of the serum sample, age, sex, age at onset of diabetes, and stage of diabetic kidney disease (HR 1.99 [1.34-2.94], P=0.001). After the inclusion of further dynamic covariates, the association was no longer significant. In study III, however, endotoxemia was an independent risk factor for incident severe diabetic retinopathy (**Table 11B**) despite the inclusion of both static and dynamic risk factors (HR 1.58 [95% CI: 1.05-2.37], P=0.03). In the sensitivity analyses, adjusting for prospective background retinopathy scores in a time-dependent model, as well as age, sex and duration of diabetes, LPS retained its significance as a risk factor for incident severe diabetic retinopathy (HR 1.63 [1.02-2.60], P=0.04).

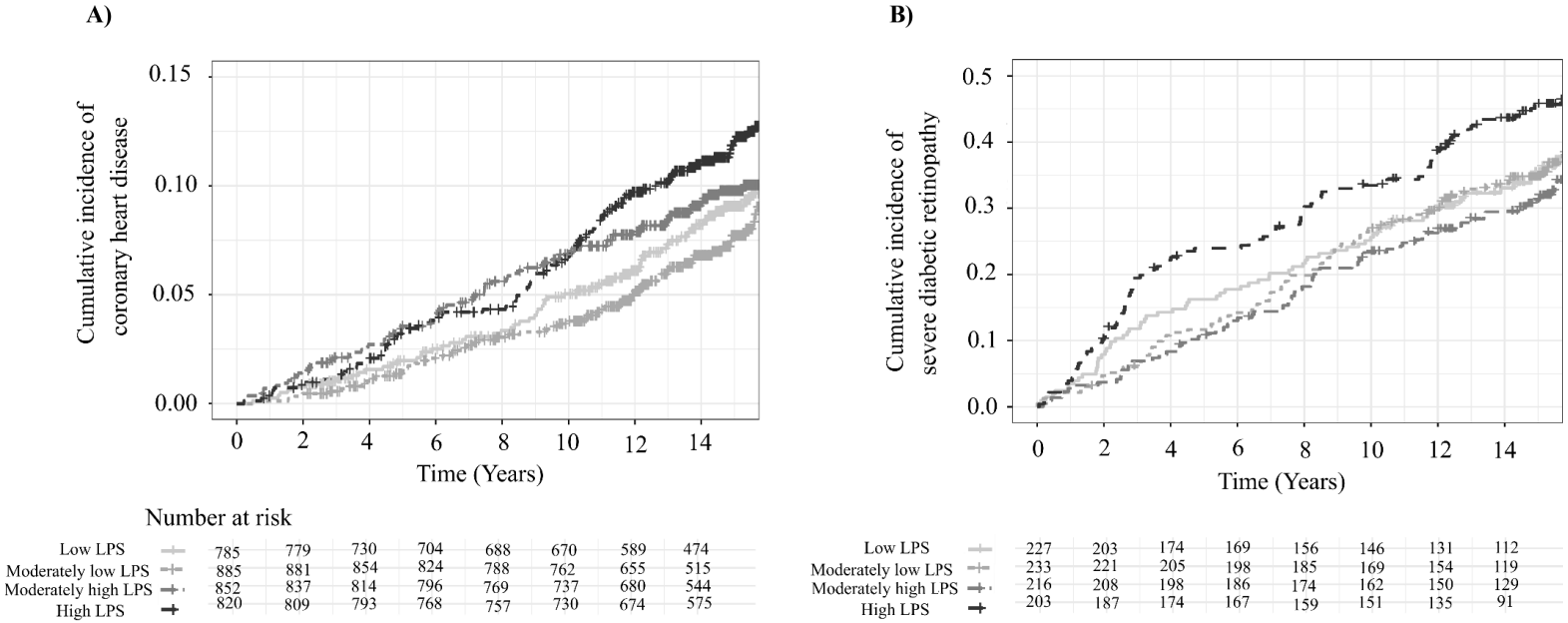


Figure 13. *Kaplan–Meier cumulative incidence curves for A) coronary heart disease, and B) severe diabetic retinopathy, over a follow-up of 15 years in individuals with type 1 diabetes. Individuals were stratified according to the interquartile range of LPS-activity, into: High LPS (LPS≥0.73), moderately high LPS (LPS≥0.51-<0.73), moderately low LPS-activity (LPS≥0.36-0.51), and finally low LPS-activity (LPS<0.36 EU/ml).*

Table 11. Results from multivariable Cox proportional hazards regression models, demonstrating LPS-activity as a risk factor for A) incident coronary heart disease, and B) incident severe diabetic retinopathy, in FinnDiane subjects with type 1 diabetes.

A.

Confounders included	HR (95% CI) for LPS-activity	P
Model 1: Freeze time	1.53 (1.07-2.18)	0.02
Model 2: Age, sex, age at onset of diabetes, stage of DKD, freeze time	1.99 (1.34-2.94)	0.001
Model 3: Age, sex, age at onset of diabetes, non-HDL, eGFR, HbA_{1c}, WHR, systolic blood pressure, history of smoking, stage of DKD at baseline, freeze time	1.37 (0.87-2.14)	0.17

B.

Confounders included	HR (95% CI) for LPS-activity	P
Model 1: Freeze time	2.22 (1.53-3.20)	<0.0001
Model 2: Age, sex, duration of diabetes, freeze time	2.77 (1.92-3.99)	<0.0001
Model 3: Age, sex, duration of diabetes, LDL, eGFR, HbA_{1c}, BMI, systolic blood pressure, history of smoking, freeze time	1.58 (1.05-2.37)	0.029

HR indicates hazard ratio; LPS-activity, bacterial lipopolysaccharide activity; HbA_{1c}, glycated haemoglobin; non-HDL, non-high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; WHR, waist-hip ratio; DKD, diabetic kidney disease; and BMI, body-mass index.

5.8 Common genetic variants associated with antibiotic purchase frequency in diabetes

In study IV, our aim was to identify single nucleotide polymorphisms associated with the infection susceptibility risk score (logarithmically transformed mean number of antibiotic purchases per follow-up year, further adjusted by age, sex, age at onset of diabetes, and long-term glycaemic control) for individuals with both type 1 and type 2 diabetes, using a GWAS approach. In the meta-analysis, where both the GWAS results from the FinnDiane and the DIREVA cohorts were included, we discovered, in total, 21 loci, where the top variants in the loci reached a suggestive P-value of $<10^{-5}$) (**Figure 14**). The lead locus was located on chromosome 2, where the lead variant rs62192851, reached a significance level of $P= 2.23\times10^{-7}$. This locus was in close proximity (<500 kB) to numerous genes (**Figure 15**) with

potential association to the phenotype. Interestingly, although our aim in study IV was to search for variants associated with an increased susceptibility to bacterial infections, the effect allele of the top variant discovered in the meta-analysis had a negative effect size (β:-0.13 [95% CI: -0.18 to -0.08]) and was, conversely, associated with a reduced infection frequency. The minor allele frequency of the top variant was 0.09.

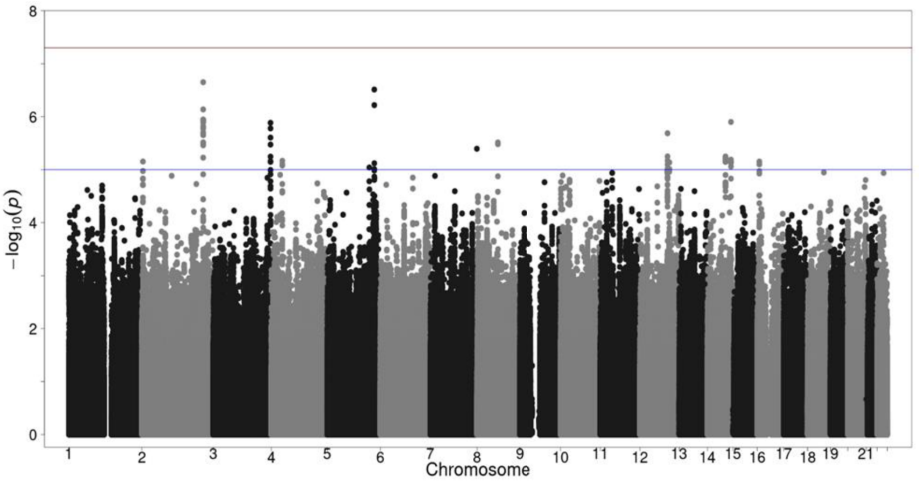


Figure 14. *Results of the meta-analysis performed on the FinnDiane (Type 1 diabetes) and the DIREVA (all types of diabetes) cohorts presented as a Manhattan plot. The red line indicates threshold for genome-wide significance (*5×10^{-8}*), while the blue line indicates suggestive significance (*10^{-5}*). The top variant is visibly located on chromosome 2.*

The narrow-sense heritability of the infection susceptibility risk score

Using a genetic relationship matrix of unrelated individuals in a mixed linear model, we calculated the proportion of the variance in the infection susceptibility risk score attributable to additive genetic factors to be 16.0% (Standard error: 0.08, P=0.02).

The effect of the lead variant's genotype on antibiotic purchase frequency

To further explore the association between our lead signal on chromosome 2 and infection susceptibility, we stratified the FinnDiane subjects into three groups based on their genotype of the lead SNP, rs62192851, into: homozygotic carriers of the reference allele, heterozygotic carriers of the effect allele and finally, homozygotic carriers of the effect allele, and investigated differences in antibiotic purchase frequencies between the groups. Here, we observed significantly lower antibiotic purchase frequencies in a dose-dependent manner with increasing numbers of effect alleles. Individuals with homozygotic carrier status of the effect alleles (N=44) had a 37% lower mean annual antibiotic purchase

frequency per subject as compared with the individuals carrying two reference alleles (N=4,231): 0.38 [IQR: 0.22-0.90] vs. 0.60 [0.30-1.20], P=0.01). Smaller differences, although still significant, were observed between heterozygotic carriers of one effect allele (N=817) and the homozygotic carriers of the reference allele; the former group had an 8% lower mean annual purchase rate per subject (0.55 [0.29-1.05], P=0.01), compared with the latter group.

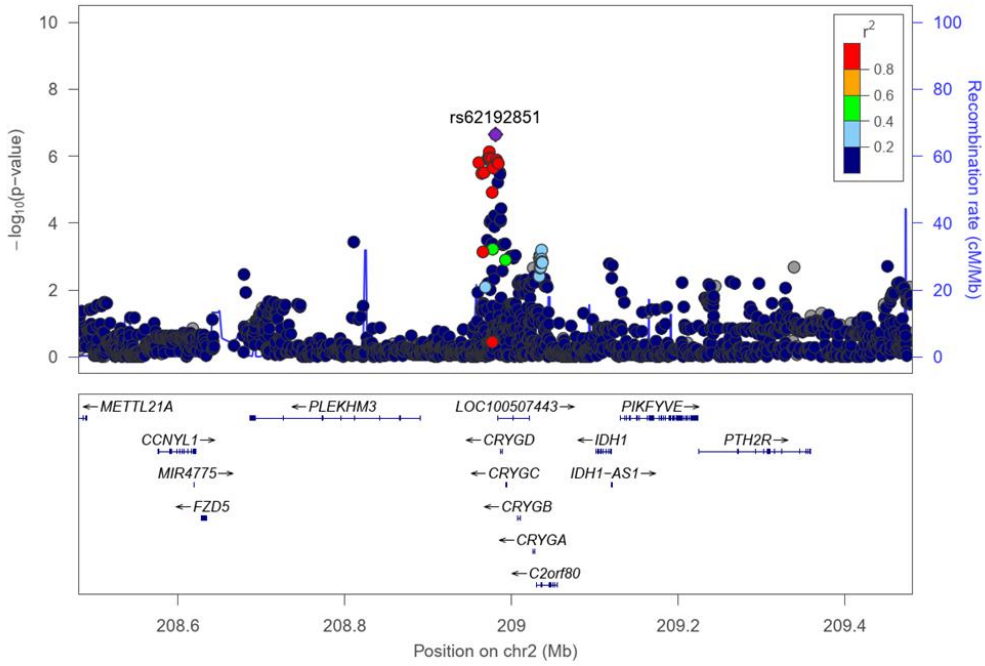


Figure 15. *Regional locus zoom plot of the lead locus located on chromosome 2 for the infection susceptibility risk score. The lead variant rs62192851 is indicated by a purple square and genes within 500 kB visible beneath the plot.*

Replication analysis:

All variants achieving a suggestive level of significance ($P<10^{-5}$) in the meta-analysis were tested for replication in the ANDIS cohort of subjects with type 1 or type 2 diabetes as well as in the FinnGen cohort of non-diabetic individuals. Although the top variant from the meta-analysis, rs62192851, failed to replicate in either replication cohort (ANDIS, P=0.60; FinnGen, P=0.52), two other variants (rs6727834 and rs10188087) from the lead locus in high linkage disequilibrium with the lead variant were nominally replicated in the FinnGen cohort (P=0.03 and P=0.04, respectively). These variants did not replicate in the ANDIS cohort (P=0.99 and P=0.95, respectively), suggesting the finding of the

association between the lead locus in meta-analysis and infection susceptibility to be more common in Finnish individuals and potentially not being limited to individuals with diabetes.

Pathway analysis

The top biological pathway enriched for association signal with our infection susceptibility risk score was the "IRAK1 recruit IKK complex" pathway ($P=5.9\times10^{-4}$). Although this finding was left as statistically suggestive after correction for multiple testing ($P_{thresh}=2.9\times10^{-5}$), the biological function of the pathway was closely linked to infections.

IRAK1 (Interleukin 1 Receptor Associated Kinase 1) is a serine-threonine kinase, the purpose of which is to initiate innate immunity and inflammatory reactions through the interleukin-1 (IL-1) as well as the TLR signalling pathways. Upon activation of either the TLR or IL-1 surface proteins, e.g., by LPS, an immunologic reaction is initiated resulting downstream in the activation of the NF-κB pathway through the IκB kinase (IKK)-complex, as well as the MAPK pathway. This results in an initiation of an inflammatory response (**Fig 16**).

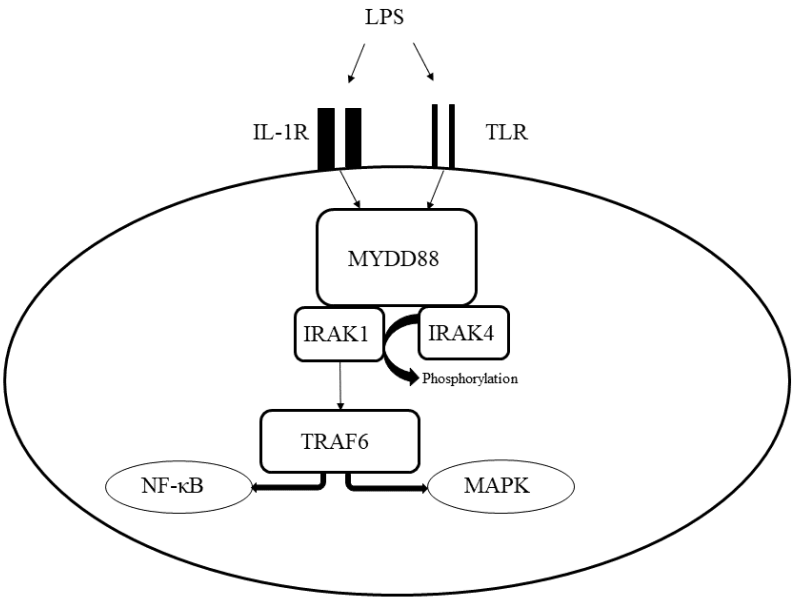


Fig 16. *The function of IRAK1. Upon stimulation of either the interleukin 1-receptor (IL-1R) or the toll-like receptor (TLR) on the surface of the cell by LPS, the adaptor protein MyDD88 is recruited to the cytosolic domain of the receptors. Here, IRAK4 is recruited, which in turn leads to IRAK1 binding to the myddosome complex as well. IRAK4 phosphorylates IRAK1, leading to the activation and migration of IRAK1 to the TRAF6-protein. This binding of IRAK1 to TRAF6 activates TRAF6, which then drives the stimulation of the NF-κB-pathway through the IκB kinase (IKK)-complex, as well as the MAPK-pathway, leading to an inflammatory response.*

6. DISCUSSION

In this thesis and in studies I-IV, we investigated the frequency of bacterial infections in individuals with type 1 diabetes and how the infections associated with micro- and macrovascular complications of diabetes. We further studied the potential genetic factors affecting the susceptibility to bacterial infections in diabetes.

6.1 Overview of the results in study I-IV

Our results show, first and foremost, that bacterial infections are significantly more common in type 1 diabetes as compared with the general population: roughly two times, when not considering comorbidities. When we compared individuals with type 1 diabetes without signs of diabetic kidney disease with individuals from the general population with similar age and sex, while adjusting for comorbidities, the individuals with type 1 diabetes were prescribed antibiotics 1.5-times more frequently, compared to the NDCs.

Both the antibiotic purchase rates as well as the rates of bacterial infections treated in hospitals correlated with the severity of diabetic kidney disease, where increasing rates of albuminuria were associated with increasing rates of bacterial infections. An increased risk of bacterial infection also correlated with long-term hyperglycaemia, where each 1%-increase in the HbA_{1c} was associated with a 6-10% increase in antibiotic purchases annually, depending on the stage of diabetic kidney disease. Antibiotic purchase frequency and high levels of endotoxemia were found to be independent risk factors for incident coronary heart disease and severe diabetic retinopathy, respectively, despite rigorous adjustment for other risk factors of the complications.

In our GWAS analysis in study IV, numerous loci were found to suggestively associate with antibiotic purchase frequencies, notably, a locus on chromosome 2, in which the top variant, rs62192851, reached a significance level of $P=2.23\times10^{-7}$. In the FinnDiane individuals, the number of effect alleles of rs62192851, was dose-dependently associated with lower antibiotic purchase frequencies. Two variants in high linkage disequilibrium with the lead variant were found to nominally replicate in a Finnish non-diabetic cohort, although no variants replicated in a Swedish diabetic cohort. Pathway analysis further suggested that this effect was mediated by the IRAK1 mediated NF-κB activation through IKK complex recruitment-pathway.

6.2 Strengths and limitations of the register data

Our solution of using register-based data for mapping bacterial infections and assessing infection frequencies in the FinnDiane participants proved to be an effective method. It allowed for the retrospective assessment of infections treated in Jan 1st 1965 up to Dec 31st 2015, although as we only used data from the same time period as the Finnish National Drug Prescription Register (Jan 1st 1995-Dec 31st 2015), we ended up not using data prior to 1995. The extensive register data resulted in follow-

up periods of considerable length and the possibility of investigating the effects of chronic complications developing or progressing over long periods of time. Importantly, it also presented a way of using longitudinal data on infections to estimate their associations with the chronic complications. As FinnDiane-subjects are prospectively followed and re-examined, we were able to further incorporate longitudinal data on clinical risk factors for chronic complications and adjust our models accordingly, increasing the accuracy of our estimates while minimizing the effect of confounders.

The register-based data, however, contains limitations due to its nature. The main limitation in the Finnish drug prescription purchase register is the lack of data on indications. In the vast majority of all antibiotic purchases in the register, excluding antibiotics prescribed for urinary tract infections, the indication and underlying infection for which the antibiotics were prescribed is unknown. This hampered our ability to study the associations of diabetic complications with specific infection foci or bacteria. It is also important to acknowledge the fact that antibiotic treatments are sometimes prescribed for viral infections due to lacking diagnostics or as a precaution to prevent secondary bacterial infections in high-risk groups. As we were unable to determine the proportion of these viral infections that were treated with antibiotics in the data, it challenged our solution of using antibiotic prescription purchases as proxies for bacterial infections. Furthermore, a significant potential confounder in the drug prescription register is the presence of prophylactic antibiotic treatments in the data, as the register also includes prescriptions for prophylactic treatment of recurring urinary tract infections or other bacterial infection prophylaxis. However, we deemed the inclusion of these prophylactic antibiotic treatments necessary, as these recurring infections may reflect an increased bacterial infection and inflammatory burden in the affected individuals. As we also had no information *a priori* on the causality behind the association between infections and diabetic complications, and the possibility that the antibiotic treatments themselves could play a role in the pathogenesis of the diabetic complications, exclusion of these recurrent antibiotic treatments could have resulted in a selection bias. Furthermore, the definition of prophylactic antibiotics would have been based on arbitrary thresholds for the daily defined dose of each antibiotic compound, which would have also been a considerable investigator bias.

In the Hospital Discharge register, the main limitation is the lack of causality in the diagnoses. The diagnoses in the register are only discharge diagnoses and therefore the register does not specify whether the infection was the cause of the hospitalisation, merely a by-stander and a coincidental finding at the time of hospitalisation, or even a condition the individual developed within the hospital. In Study I, where we compared infection frequencies, and specifically, hospitalisation rates between non-diabetic individuals and individuals with type 1 diabetes, this limitation may, in part, explain the difference observed between individuals with type 1 diabetes and NDCs, as diabetes itself generally lowers the threshold for hospitalisation. Diabetes may also potentially lower the threshold of prescribing antibiotics, which may also be a reason for the observed differences in the antibiotic prescription purchase frequencies present in the drug prescription register.

We observed that out of all bacterial infection events (either bacterial infections treated in hospitals or antibiotic purchases in outpatient care) during 1996-2015, only 8% were infections treated within hospitals, while 92% were infections treated with antibiotic purchases in outpatient care. In study I, as we calculated the annual infection rate per 1,000 individuals or per 1,000 person years and compared this exposure between groups, the covariate was sound and the analyses were feasible. However, in study II and III, analyses using the hospital discharge register data on inpatient bacterial infections proved infeasible. Although these analyses were initially attempted, the rarity of hospital treated bacterial infections introduced a substantial number of excess zeroes, in addition to violating the proportional hazards assumption in the Cox regression models, leading to time-dependency issues. In an alternative approach, where the inpatient infections, together with the antibiotic purchases, were pooled into one infection covariate, they yielded little gain in statistical power, while simultaneously introducing more heterogeneity in the covariate and phenotype. For these reasons, only the data on antibiotic purchases were used to reflect bacterial infections as risk factors for diabetic complications in studies II-III. Regarding study IV, in the FinnDiane cohort the analyses were initially conducted by including the data on bacterial infections treated in hospitals as infection events in the FinnDiane cohorts. However, data on bacterial infections treated within hospitals was not available for the DIREVA study, only data on antibiotic purchases. In order to harmonize the methodology and phenotype between the cohorts, the hospital discharge register data was also excluded from the FinnDiane-phenotype. Notably, this change in phenotype had very little effect on the results in the GWAS performed in the FinnDiane cohort.

6.3 Methodological strengths and limitations

Infections as risk factors for incident diabetic complications in Cox proportional hazards models: Time-dependency issues

In study II and III we used Cox regression analysis to investigate antibiotic purchases as risk factors for incident diabetic complications. One fundamental rule regarding the eligibility of Cox proportional hazards regression models, is that the hazard ratio of the covariates does not vary during the follow-up. If the magnitude of the risk is dependent of time, it violates the proportional hazards assumption and the covariate is considered time-dependent. Theoretically, it is possible that the effect of bacterial infections on the risk of diabetic complications is greater, the closer the infection is to the complication outcome. Therefore, as such, infections are potential time-dependent covariates when applied as covariates to Cox proportional hazards regression models. Ideally, this association between the infection and the complication outcome would be investigated using a time-dependent model, where the follow-up would be divided into specific time-windows and the effect of the infection on the outcome would then be assessed in step-wise functions. This was extensively attempted in the analysis of the antibiotic data in study II and III, using different methods demonstrated in earlier publications[279]. However, the

analysis approach proved to be infeasible due to restrictions imposed by the nature of the data as the value of each antibiotic purchase in these analyses was always one at different time points, resulting in a covariate with no variance.

Our solution to this limitation was two-fold: first, instead of using each infection event individually in the analysis, we adjusted the covariate directly for time, as suggested previously by Zhang et al.[279], and calculated a mean infection frequency over the whole follow-up. This had some important ramifications, specifically, regarding the interpretation of the hazard ratios in the models. By directly adjusting the covariate for time, the hazard ratios reflect the average annual exposure during the follow-up. As such, although the hazard ratio in the model of the mean number of antibiotic purchases per follow-up year describes how much each annual purchase increases the risk of the outcome, the results may not potentially be applicable to estimate the association between a single infection and an incident complication outcome within short time frames, and must instead be interpreted as an infection frequency over longer periods of time.

Our second solution to the potential time-dependency issue of infections as risk factors for incident complication events, was to calculate and compare the total numbers of antibiotic purchases annually before the incident events in specific case-control pairs. This analysis approach yielded interesting results as we observed significant differences in antibiotic purchase frequencies between the case-controls groups even up to two years before the events, further raising questions regarding the mechanisms behind this association. The limitation in this approach, however, was the loss of statistical power, as the need for similar age, sex, and duration of diabetes in the case-control pairs reduced the number of available participants in the analyses. Therefore, whether the non-significant differences in antibiotic purchase frequencies between the case-control groups visible in earlier years before the events are indeed non-significant or simply due to insufficient statistical power, is unknown.

Importantly, the adjustment for the follow-up time of the antibiotic purchases in the analyses addressed significant potential limitations. Direct comparison of the total antibiotic purchases between individuals with and without incident diabetic complications, without taking the length of follow-up into consideration, would have resulted in a bias, since individuals with diabetic complications had markedly shorter follow-ups and therefore smaller numbers of total purchases due to less time to purchase antibiotics. Adjusting the total numbers of purchases for the follow-up time effectively dealt with this bias and allowed the direct comparison of purchase frequencies between the groups. In terms of time-dependency, the time-adjusted infection covariate also proved to be sound, as the hazard ratio displayed no significant variance over time.

Adjusting for diabetic kidney disease in study II – strength or limitation?

As we observed a significant association between diabetic kidney disease and bacterial infection frequencies in study I, we deemed it necessary to adjust our regression models for diabetic kidney disease when we investigated the association between bacterial infections and coronary heart disease in study II and inserted both stage of diabetic kidney disease at baseline as well as eGFR as confounders into the Cox regression models. We further excluded individuals who developed ESRD from the analyses altogether and additionally, excluded individuals where progression of diabetic kidney disease was observed during the follow-up in the sub-analyses in specific stages of diabetic kidney disease. Although this maximized our adjustment for diabetic kidney disease, it may also have created a selection bias of the study cohort, as we excluded individuals where the diabetic kidney disease progresses more rapidly and where antibiotic purchases are potentially more frequent, compared to individuals with sustained stages of diabetic kidney disease. In addition, as diabetic kidney disease and coronary heart disease are strongly associated with one another and share similar risk factors, it is possible that the adjustment for diabetic kidney disease also adjusted, to some extent, for overall vascular damage. Therefore, these factors may have diluted the results in study II, and the association between bacterial infections and coronary heart disease may be even stronger than the one reported. None the less, we would argue that the adjustment for diabetic kidney disease was important, as diabetic kidney disease is a major risk factor for coronary heart disease, and therefore, our findings support the theory that the infections indeed are independent risk factors of coronary heart disease outside of their association to diabetic kidney disease.

In study III, we took a different approach when adjusting the associations between severe diabetic retinopathy and bacterial infection frequencies for diabetic kidney disease. To avoid the potential selection bias we experienced in study II, we included all individuals with normal AER, microalbuminuria or macroalbuminuria at baseline, regardless of the potential progression of diabetic kidney disease during follow-up. Also, instead of excluding individuals progressing to ESRD, we censored the follow-up when the individuals developed ESRD instead. Furthermore, only eGFR was included as a covariate in the Cox regression models to adjust for renal function. As both diabetic kidney disease and retinopathy are microvascular by nature, the adjustment for diabetic kidney disease could theoretically have adjusted the regression models for certain degrees of diabetic retinopathy as well. Interestingly though, quite similar hazard ratios were observed in study II and III for both antibiotic purchases and endotoxemia as risk factors for coronary heart disease and diabetic retinopathy, respectively, despite the differing approaches to adjust for kidney disease. Of note, the effect of diabetic kidney disease in the Cox proportional hazards ratio in study III, was investigated and published in the supplemental material.

The phenotype of the GWAS analyses

A noteworthy limitation in study IV is the adjustment of glycaemic control in the definition of the infection susceptibility risk score. Poor glucose control has previously been demonstrated as a risk factor for infections, and in study I, we observed a significant association between poor glycaemic control and bacterial infection frequencies. As HbA_{1c} can be considered an environmental factor affecting infection susceptibility, we used the mean HbA_{1c} during follow-up as a covariate in the GWAS analyses. This adjustment also, in part, adjusted the GWAS results for diabetic kidney disease, as HbA_{1c} strongly correlates with diabetic kidney disease. However, it is possible that hyperglycaemia is a major mechanistic factor affecting infection susceptibility in diabetes, as it also is one of the main clinical features differentiating individuals with and without diabetes. Therefore, this adjustment may have diluted and/or confounded our results.

Another factor potentially affecting the significance of our results is the heterogeneity of the phenotype. We included all antibiotic purchases under the ATC-code of J01 as well as several different types of diabetes in the analysis. Although this increased the number of events as well as the cohort sizes, it may have caused more variance in the parameters, and therefore, conversely decreased the statistical power. On the other hand, as all types of orally administered antibiotics were included in the analyses, the association signals observed in the meta-analysis may reflect immunologic checkpoints relevant in bacterial infections caused by several different bacterial strains.

6.4 Comparison of results to previous research

We observed a roughly two-fold greater bacterial infection frequency in individuals with type 1 diabetes, compared to the general population. This is in line with a previous study, where the risk of hospitalisation due to a bacterial infection was between 1.5-2-fold greater in individuals with type 1 diabetes[8]. Other studies have reported greater incidence rates in hospitalisation due to infections: Carey et al. found that individuals with type 1 diabetes had closer to four-fold greater numbers of infection-related hospitalisations compared to NDCs[191], similar to the incidence rates that we observed in individuals with albuminuria in study I. Although, notably, the study by Carey et al. did not adjust for albuminuria, but only chronic kidney disease as a binomial categorical variable, potentially explaining the increased risk in their study if the individuals had advanced albuminuria without markedly lower glomerular filtration rates.

We found that poor glycaemic control correlated with antibiotic purchase frequencies as well as hospitalisations due to bacterial infections. More specifically, we observed a 6-10% increase in antibiotic purchases with each 1% increase in baseline HbA_{1c}. Very similar associations were published by Critchley et al. in 2018[190], where the authors found that during a 2-year follow-up of 104,717 individuals with diabetes in the United Kingdom, each increase of HbA_{1c} was associated with a 3-10% increase in infections treated with antibiotics, after adjustment for age, sex, smoking, body mass index,

deprivation quintile, and type of diabetes. These results, together with the results from study I, indicate that there is indeed a strong association between glycaemic control and infection frequency in diabetes.

High levels of endotoxemia have previously been reported to associate with incident cardiovascular disease. Pussinen et al. found that the adjusted hazard ratio for endotoxin/HDL-ratio for incident cardiovascular disease was 1.92 (95% CI: 1.19-3.08) in a Finnish random sample[25]. In our study in individuals with type 1 diabetes, we found very similar hazard ratios for LPS for incident coronary heart disease in type 1 diabetes (HR adjusted for static confounders: 1.99 [1.34-2.94]). As the hazard ratios are quite similar in magnitude in two different cohorts, our results validate and strengthen the hypothesis that endotoxemia is indeed a relevant risk factor for cardiovascular disease. Of particular interest, we observed similar hazard ratios for endotoxemia as a risk factor for incident severe diabetic retinopathy (HR adjusted for static confounders: 2.77 [1.92-3.99], HR adjusted for static as well as dynamic confounders 1.58 [1.05-2.37]). As this association and finding was novel to our knowledge, no previously published reports were available for comparison.

6.5 Infections, the risk of developing chronic diabetic complications and causality

Perhaps the most significant limitation in this thesis and in Study I-IV is the study setting. As all of our studies were observational by nature, we cannot draw conclusions on the causality behind our findings. This especially concerns Study II and III, where we could not ascertain whether the infection covariates directly raise the risk of complications, or potentially through one or even several mediators.

One important unclear aspect in these mechanistic questions is the interplay between bacterial infections, antibiotic treatments, and endotoxemia (**Fig 17**). Studies have found that dysbiosis in the gut may increase LPS-activity in serum[237]. As antibiotics may cause dysbiosis to different degrees, depending on the spectrum of the antibiotic, they may, therefore, also theoretically increase LPS-activity and potentially increase the risk of developing a diabetic complication through the induction of endotoxemia. Although, notably, in Study II, we assessed this interplay to a certain degree in electronically published supplemental analyses, by including both LPS-activity and antibiotic purchases into the same Cox regression model as risk factors for incident coronary heart disease and found that this inclusion had very little impact on the hazard ratio and significance of either covariate, suggesting that the effect on the risk of coronary heart disease attributable to the antibiotics and LPS arises through different mechanisms. Inflammation, both acute as well as chronic, is also a potential or even likely mediator of the increased risk of chronic complications attributable to bacterial infections. However, whether the main instigator for the potential inflammatory response is the bacterial colonisation during the infection, the immunologic inflammatory response, the resulting dysbiosis by the antibiotic treatment, the consequential endotoxemia, or a synergistic effect of all four, is unclear.

As LPS is a major driver of inflammatory response and only present in gram-negative bacteria, it is conceivable that the inflammatory response to gram-negative bacterial infections may differ from gram-

positive infections. Interestingly, in our post-hoc analysis of study II, where we included only antibiotic purchases mainly used in the treatment of urinary tract infections, the covariate presented hazard ratios equal in both significance and magnitude as the analysis where all antibiotic purchases were included. Considering that these analyses had 20% of the purchase events than the more robust and methodologically identical analysis using all antibiotics, and taking into account that the vast majority of all urinary tract infections are colonisations by gram-negative bacteria, this finding can be considered quite significant. Additionally, this also potentially demonstrates how peripheral infections may increase the risk of cardiovascular disease.

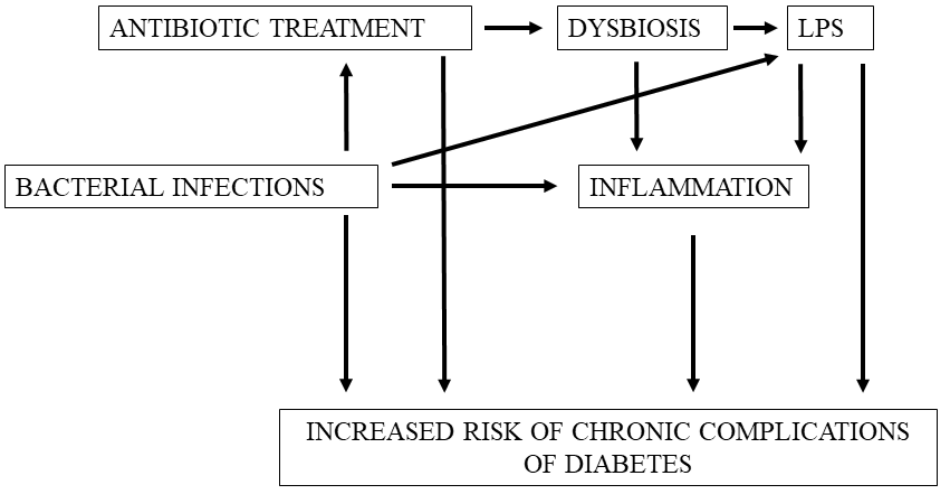


Figure 17. *Theoretical flow chart of potential causative links between bacterial infections and the development and/or progression of chronic complications of diabetes.*

Previously, clinical trials have attempted to demonstrate causative links between infections and cardiovascular disease by using antibiotic interventions in randomized clinical trials in order to reduce the risk of cardiovascular disease. As these have failed to produce consistent and reliable protective effects, some researchers have concluded that there exists no causality between infections and cardiovascular disease. The premise of these clinical trials, however, was that the antibiotic treatments would protect the individuals from cardiovascular disease and the trials failed to consider the opposite: that the antibiotic treatments might, in fact, increase the risk of cardiovascular disease. In study II, even though the antibiotic purchases were used as a proxy exposure to reflect bacterial infections, the fact remains that the discovered association was specifically between antibiotic prescription purchases and incident coronary heart disease, not recorded bacterial infections. Furthermore, in our study, these associations between antibiotic purchases and coronary heart disease were found after an extensive

follow-up period (median length of 14 years) in a well-defined and comprehensive cohort of over 3,700 individuals. Therefore, it is plausible that the non-significant associations observed between infections and cardiovascular disease in previous clinical trials can be explained by an inaccurate *a priori* hypothesis, relatively short follow-up periods in combination with a low-impact intervention, as only macrolides were used as treatment methods.

6.6 Comparison of chronic diabetic complications in regard to bacterial infections

Previous research has demonstrated close associations between the different chronic complications of diabetes to one another[280 281 282 283]. The chronological order in which the diabetic complications present themselves vary greatly between individuals. Diabetic retinopathy is usually considered the first complication to develop, and therefore, acts as a predictor for the other complications[284 285], as retinopathy reflects the presence of existing microvascular damage. It is, however, conceivable that diabetic retinopathy and diabetic kidney disease are chronologically often the first complications, simply because they are, arguably, the easiest complications to screen for. Retinal exams offer a unique, direct macroscopic examination of the vasculature bed in the retina, while simple urinary spot tests may reveal proteinuria, and blood tests may detect a reduced GFR. Corresponding clinical assessment of the other anatomical sites of diabetic complications are unavailable. In fact, the diagnosing of diabetic neuropathy and cardiovascular disease is much more challenging. The nature of diabetic neuropathy and the symptoms vary greatly between individuals, depending on which nerves and anatomical sites are affected, and the classification of the disease is more complicated, compared to the other complications. Symptoms may present as peripheral neuropathy, cardiovascular autonomic neuropathy, or simply gastrointestinal motility problems easily considered physiological or transient. Cardiovascular severe events are clinically robust evidence of cardiovascular disease, however, less severe forms of cardiovascular disease, e.g., microvascular damage in the coronary arteries, may be complicated to diagnose, as individuals with diabetes may present with atypical symptoms or even none at all. This is also potentially why the other complications are considered to predict cardiovascular disease, as it can be clinically challenging to diagnose mild forms of cardiovascular disease in diabetes even though the pathophysiological damage in the affected tissue is present, while mild forms of retinopathy and diabetic kidney disease are likely more easily discovered.

Using very similar methodologies and identical infection covariates to reflect bacterial infections in study II and III, in order to assess their role as risk factors of different diabetic complications, provided a unique opportunity to compare the magnitudes of the hazard ratios of the covariates to each other with different diabetic complications as outcomes. In our studies II-III, we found that the mean number of antibiotic purchases per follow-up year and high LPS-activity were significant risk factors for the development of coronary heart disease as well as severe diabetic retinopathy and interestingly, with remarkably similar hazard ratios. This raises the question, how similar diabetic retinopathy and

coronary heart disease are as diseases, and whether they simply are presentations and reflections of the same chronic assault of hyperglycaemia on blood vessels in different tissues and end organs in individuals with diabetes, further augmented by other risk factors such as bacterial infections and genetic predisposition to certain disease. Although the present thesis is unable to answer this question, the similar hazard ratios in the Cox regression models in study II-III indicate that the association between the complications is significant and underlines previous research that demonstrates the interplay between the different complications.

6.7 Future implications

Regarding the immediate implications to clinical practice by this thesis, even though it is uncertain at present whether the bacterial infections directly increase the risk of chronic complications of diabetes, effective preventive treatment of bacterial infections in type 1 diabetes is, and has already been recommended. Bacterial infections are potentially life-threatening conditions and the infections seem to exhibit more severe forms in type 1 diabetes, compared with the general population, including the progression to septicaemia. As to how this prevention of bacterial infections could optimally be carried out is debatable, although several methods are already in use in clinical practice. Thorough treatment of peripheral skin ulcers and optimisation of glycaemic control are routine cornerstones in the treatment of diabetes. These treatments also benefit the affected individuals by reducing overall morbidity and mortality, while likely reducing the risk of infections as well. Effective national vaccination programmes of preventable infections are recommended to individuals with diabetes, including the annual vaccines for *Streptococcus pneumoniae* and the *Influenza*-viruses. Finally, diabetic complications are complex diseases with several risk factors, including hypertension, hyperglycaemia, dyslipidaemia, and cessation of smoking, and effective treatment of these risk factors are already included in the national clinical guidelines for diabetes.

In study I, we observed that antibiotic prescriptions were much more common in individuals with type 1 diabetes, compared to the general population. This frequent use of antibiotics incurs a higher risk of colonisation by antibiotic-resistant bacteria. These concerns have previously already been voiced as antibiotic resistant strains of bacteria have been found with accelerating frequency in individuals with diabetes in India, Cameroon, Japan, and the United States[286 287]. Although, as several countries have not investigated or published data on the frequency of antibiotic-resistant strains in individuals with diabetes, the prevalence of these resistant bacterial strains is likely far greater than reported. Taking into account the accelerating resistance to antibiotics observed globally[288], antibiotic-resistant strains of bacteria are potentially a major future concern, especially in individuals with diabetes. Emerging novel therapeutic methods, including treatment with bacteriophages, may, in the future, offer alternative treatment methods of infections, potentially providing a solution to infection by antibiotic-resistant bacteria[289].

Individuals with diabetes have been shown to display higher levels of endotoxemia in serum compared to the general population. LPS is known to directly cause damage in the kidney and act as a risk factor for both diabetic kidney disease as well as coronary heart disease. In study III, we further demonstrated endotoxemia to be robustly associated with severe diabetic retinopathy. Direct LPS-antagonists, such as alkaline phosphatase therapy, have been shown to reduce sepsis-related mortality and kidney injury[290]. Countering the effects of LPS in serum in individuals with diabetes and endotoxemia might, therefore, reduce the risk of the development or progression of diabetic complications. However, this hypothesis raises the important question of the source of endotoxemia in individuals with diabetes and how concentrations of LPS act during acute bacterial infections with gram-negative bacteria: whether or not the concentrations of LPS significantly increase during gram-negative infections, and if they remain elevated after the resolution of the bacterial infection, exacerbating the inflammatory response and potentially increasing the risk of diabetic complications. Further research of the behaviour of endotoxemia might reveal novel therapeutic mechanisms and ways of reducing the risk of diabetic complications.

As diabetic complications are the major drivers of morbidity and mortality in diabetes, the more specific pathophysiologic chain of events behind the associations between bacterial infections and the emergence of chronic diabetic complications merit future mechanistic studies. Depending on these mechanisms, it is also conceivable that similar mechanisms behind the associations between infections and cardiovascular disease or chronic kidney disease could be found in the general population, further underlining the importance of future studies.

7. SUMMARY AND CONCLUSIONS

I. Individuals with type 1 diabetes are prescribed antibiotics and treated for bacterial infections within hospitals approximately, twice as frequently, compared to the general population.

II. Poor glucose control is strongly associated with more frequent antibiotic prescription purchases in individuals with type 1 diabetes.

III. Both antibiotic purchases and bacterial infections treated in hospitals correlate with the severity of diabetic kidney disease. More frequent antibiotic purchases were observed two years before, during, and 1 year after the onset of microalbuminuria.

IV. Frequent antibiotic purchases are an independent risk factor for incident coronary heart disease in individuals with type 1 diabetes as well as a strong risk factor for severe diabetic retinopathy. Conversely, endotoxemia is an independent risk factor for incident severe diabetic retinopathy, as well as a strong risk factor for incident coronary heart disease, in individuals with type 1 diabetes.

V. Common variants on chromosome 2 may be associated with a decreased infection frequency in Finnish individuals with diabetes, potentially mediated through the *IRAK1* pathway.

8. ACKNOWLEDGEMENTS

At long last I arrive at the acknowledgments, and an opportunity to express my sincerest gratitude to everyone who have participated in my research finally presents itself.

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I wish to thank professor Ilkka Pörsti and docent Reetta Huttunen for reviewing this thesis and for the valuable comments and suggestions for improvement they provided. They made a big difference.

I also wish to warmly thank all of the FinnDiane participants who selflessly offer up their time to help us collect the data we need, and for whom we're conducting our research. I sincerely hope that my past, present and future studies will benefit you.

And on to my supervisors, close colleagues and my family.

Markku, it all began with your e-mail in March 2011. At the time, you were searching for a medical student ready to undertake a project concerning infections in type 1 diabetes. When I eagerly arrived at the interview, you even proposed that said student would potentially write a scientific publication on the matter. As I had little (let's be honest, none whatsoever) understanding of what this entailed, I heartily agreed. And I'm profoundly grateful that I did. My first steps into academia and the last years have at times been exhilarating, at other times strenuous, but never dull. Having reached this milestone, I cannot thank you enough for all of the guidance, mentoring and advice you've given over the years.

Perra, you storming into our room in Biomedicum and without much warning asking "what have you learned today?!" happened more than once. How you manage to find time for discussing and contemplating my projects, while managing our study group, teaching medical students nephrology, performing clinic duty, sitting at board meetings and giving international lectures is beyond me. One of the most important lessons I picked up from you was that even though details require attention, I should never get lost in them, and remember the broad picture of what we are researching and why. To always return to the fundamental questions. These are important lessons that I hope will serve me well in all my future endeavours, and for which I am truly grateful.

Carol, even though I was unable to include you as an official thesis supervisor, you've certainly acted the part. Especially, as I was returning to FinnDiane and research in 2018 after a break of some years when I was off performing clinic duty, the frequent discussions with you and your never-ending stream of ideas and feedback were invaluable. Outside of this thesis, I dare say that my interest in academia and research are also in large part thanks to your support over the years. Thank you for everything.

Valma, you've provided me with immeasurable support over the years with statistical analyses, project planning and phenotype data. Thank you for all of your assistance in all of the studies (especially the first study which we, in the end, co-authored), your teachings about statistics and for finding the time to answer my questions. Without your and Markku's help, I would have quickly become lost in the jungle.

Asko, even though you most likely were sorely pressed for time, you participated in the production of all our studies and provided us with the insights into infectious diseases you've acquired over your long career as both a clinician and a researcher. Your feedback and ideas were invaluable from start to finish, but absolutely crucial in the design of our methodology. Thank you for being part of our team.

Niina, Anni, Erkka, Anna, Jani, Emma, Stefan and Nadja (whom I have in private unceremoniously collectively called "The IT-wizards"), we all know that I needed a lot of help with genetic analyses and learning R. A lot. Thank you once more. As is my quite established *modus operandi* at this point, I'll offer freshly ground and brewed coffee in return for your mentoring.

My fellow co-authors in study III and IV: Kustaa, thank you for all of your contributions and suggestions, they improved the analyses and paper significantly. Tiinamaija, Leif, Annemari, Emma, Rashmi, Dina, and Iiro, it was a great pleasure to work with you and thank you ever so much for your hard work.

Without the excellent FinnDiane physicians and study nurses all of my studies would have been impossible. Thank you for all your efforts and accomplishments. Anna, Kirsi and Jaana, you're an absolute joy to work with and I'm looking forward to all of our ongoing and future projects. Can't wait.

Mom, Dad, you've played a pivotal role in my choice of career, even though neither of you are perhaps aware of this, or well-versed in medicine for that matter (no Mom, even though you argue otherwise, it's true). Mom, you always seemed to hold people in health care in high esteem and I guess at some point that rubbed off on me. You're one of the kindest and most empathic people I have and most likely will ever know, and if I know anything about the aforementioned qualities, I learned it from you. Dad, you always advocated for dedication and commitment to whatever task I put my mind to. This taught me perseverance, which I can easily say is one of the fundamental reasons for me being where I am today. You both always spurred me on, regardless of the height of the hurdles ahead, and your support,

joined with simple curiosity, led me mostly through chance into medicine and further into research. For the life lessons and advice, you've provided me with, I'm eternally grateful.

And at the very end I come to my family. Mia, words fail me at this point, as they are not nearly enough to convey my mixed feelings of respect, pride, and love for you. It will have to do with a purposefully inaccurate quote by Kiersten White: "*In a hundred lifetimes and in all of the worlds, I would find you, and I would choose you.*" My children, you are the single greatest joy and the most precious thing in my life, you continue to amaze me. I love you dearly. Finally, it is done.

"Only in silence the word,

Only in dark the light,

Only in dying life:

Bright the hawk's flight

On the empty sky."

— Ursula K. Le Guin, excerpt from The Creation of Éa.

9. APPENDIX

List of physicians and nurses at each of the FinnDiane centres participating in patient recruitment and characterization.

The Finnish Diabetic Nephropathy Study Centers

Anjalankoski Health Center	S.Koivula, T.Uggeldahl
Central Finland Central Hospital, Jyväskylä	T.Forslund, A.Halonen, A.Koistinen, P.Koskiaho, M.Laukkanen, J.Saltevo, M.Tiihonen
Central Hospital of Åland Islands, Mariehamn	M.Forsen, H.Granlund, A.-C.Jonsson, B.Nyroos
Central Hospital of Kanta-Häme, Hämeenlinna	P.Kinnunen, A.Orvola, T.Salonen, A.Vähänen
Central Hospital of Kymenlaakso, Kotka	R.Paldanius, M.Riihelä, L.Ryysy
Central Hospital of Länsi-Pohja, Kemi	H.Laukkanen, P.Nyländen, A.Sademies
Central Ostrobothnian Hospital District, Kokkola	S.Anderson, B.Asplund, U.Byskata, P.Liedes, M.Kuusela, T.Virkkala
City of Espoo Health Center:	
Espoonlahti	A.Nikkola, E.Ritola
Tapiola	M.Niska, H.Saarinen
Samaria	E.Oukko-Ruponen, T.Virtanen
Viherlaakso	A.Lyytinen
City of Helsinki Health Center:	
Puistola	H.Kari, T.Simonen
Suutarila	A.Kaprio, J.Kärkkäinen, B.Rantaeskola
Töölö	P.Kääriäinen, J.Haaga, A-L.Pietiläinen
City of Hyvinkää Health Center	S.Klemetti, T.Nyandoto, E.Rontu, S.Satuli-Autere
City of Vantaa Health Center:	
Korso	R.Toivonen, H.Virtanen
Länsimäki	R.Ahonen, M.Ivaska-Suomela, A.Jauhiainen
Martinlaakso	M.Laine, T.Pellonpää, R.Puranen
Myyrmäki	A.Airas, J.Laakso, K.Rautavaara
Rekola	M.Erola, E.Jatkola
Tikkurila	R.Lönnblad, A.Malm, J.Mäkelä, E.Rautamo
Heinola Health Center	P.Hentunen, J.Lagerstam
Helsinki University Central Hospital, Department of Medicine, Division of Nephrology	M.Feodoroff, D.Gordin, O.Heikkilä, K.Hietala, J.Fagerudd, M.Korolainen, L.Kyllönen, J.Kytö, S.Lindh, K.Pettersson-Fernholm, M.Rosengård-Bärlund, A.Sandelin, L.Thorn, J.Tuomikangas, T.Vesisenaho, J.Wadén
Herttoniemi Hospital, Helsinki	V.Sipilä
Hospital of Lounais-Häme, Forssa	T.Kalliomäki, J.Koskelainen, R.Nikkanen, N.Savolainen, H.Sulonen, E.Valtonen
Hyvinkää Hospital	L. Norvio, A.Hämäläinen
Iisalmi Hospital	E.Toivanen
Jokilaakso Hospital, Jämsä	A.Parta, I.Pirttiniemi
Jorvi Hospital, Helsinki University Central Hospital	S.Aranko, S.Ervasti, R.Kauppinen-Mäkelin, A.Kuusisto, T.Leppälä, K.Nikkilä, L.Pekkonen
Jyväskylä Health Center, Kyllö	K.Nuorva, M.Tiihonen
Kainuu Central Hospital, Kajaani	S.Jokelainen, K.Kananen, M.Karjalainen, P.Kemppainen, A-M.Mankinen, A.Reponen

	M.Sankari
Kerava Health Center	H.Stuckey, P.Suominen
Kirkkonummi Health Center	A.Lappalainen, M.Liimatainen, J.Santaholma
Kivelä Hospital, Helsinki	A.Aimolahti, E.Huovinen
Koskela Hospital, Helsinki	V.Ilkka, M.Lehtimäki
Kotka Health Center	E.Pälikkö-Kontinen, A.Vanhanen
Kouvola Health Center	E.Koskinen, T.Siitonen
Kuopio University Hospital	E.Huttunen, R.Ikäheimo, P.Karhapää, P.Kekäläinen, M.Laakso, T.Lakka, E.Lampainen, L.Moilanen, S. Tanskanen, L.Niskanen, U.Tuovinen, I.Vauhkonen, E.Voutilainen
Kuusamo Health Center	T.Kääriäinen, E.Isopoussu
Kuusankoski Hospital	E.Kilkki, I.Koskinen, L.Riihelä
Laakso Hospital, Helsinki	T.Meriläinen, P.Poukka, R.Savolainen, N.Uhlenius
Lahti City Hospital	A.Mäkelä, M.Tanner
Lapland Central Hospital, Rovaniemi	L.Hyvärinen, K.Lampela, S.Pöykkö, T.Rompasaari, S.Severinkangas, T.Tulokas
Lappeenranta Health Center	P. Erola, L.Härkönen, P.Linkola, T.Pekkanen, I.Pulli, E.Repo
Lohja Hospital	T.Granlund, K.Hietanen, M.Porrassalmi, M.Saari, T.Salonen, M.Tiikkainen,
Länsi-Uusimaa Hospital, Tammisaari	I.-M.Jousmaa, J.Rinne
Loimaa Health Center	A.Mäkelä, P.Eloranta
Malmi Hospital, Helsinki	H.Lanki, S.Moilanen, M.Tilly-Kiesi
Mikkeli Central Hospital	A.Gynther, R.Manninen, P.Nironen, M.Salminen, T.Vänttinen
Mänttä Regional Hospital	I.Pirttiniemi, A-M.Hänninen
North Karelian Hospital, Joensuu	U-M.Henttula, P.Kekäläinen, M.Pietarinen, A.Rissanen, M.Voutilainen
Nurmijärvi Health Center	A.Burgos, K.Urtamo
Oulaskangas Hospital, Oulainen	E.Jokelainen, P-L.Jylkkä, E.Kaarlela, J.Vuolaspuro
Oulu Health Center	L.Hiltunen, R.Häkkinen, S.Keinänen-Kiukaanniemi
Oulu University Hospital	R.Ikäheimo
Päijät-Häme Central Hospital	H.Haapamäki, A.Helanterä, S.Hämäläinen, V.Ilvesmäki, H.Miettinen
Palokka Health Center	P.Sopanen, L.Welling
Pieksämäki Hospital	V.Sevtsenko, M.Tamminen
Pietarsaari Hospital	M-L.Holmbäck, B.Isomaa, L.Sarelin
Pori City Hospital	P.Ahonen, P.Merisalo, E.Muurinen, K.Sävelä
Porvoo Hospital	M.Kallio, B.Rask, S.Rämö
Raahe Hospital	A.Holma, M.Honkala, A.Tuomivaara, R.Vainionpää
Rauma Hospital	K.Laine, K.Saarinen, T.Salminen
Riihimäki Hospital	P.Aalto, E.Immonen, L.Juurinen
Salo Hospital	A.Alanko, J.Lapinleimu, P.Rautio, M.Virtanen
Satakunta Central Hospital, Pori	M.Asola, M.Juhola, P.Kunelius, M.-L.Lahdenmäki, P.Pääkkönen, M.Rautavirta
Savonlinna Central Hospital	T.Pulli, P.Sallinen, M.Taskinen, E.Tolvanen, T.Tuominen, H.Valtonen, A.Vartia, S-L.Viitanen
Seinäjoki Central Hospital	O. Antila, E.Korpi-Hyövälti, T.Latvala, E.Leijala, T.Leikkari, M.Punkari N.Rantamäki, H.Vähävuori

South Karelia Central Hospital, Lappeenranta	T.Ensala, E.Hussi, R.Härkönen, U.Nyholm, J.Toivanen
Tampere Health Center	A.Vaden, P.Alarotu, E.Kujansuu, H.Kirkkopelto-Jokinen, M.Helin, S.Gummerus, L.Calonius, T.Niskanen, T.Kaitala, T.Vatanen
Tampere University Hospital	P. Hannula, I.Ala-Houhala, R.Kannisto, T.Kuningas, P.Lampinen, M.Määttä, H.Oksala, T.Oksanen, A.Putila, H.Saha, K.Salonen, H.Tauriainen, S.Tulokas
Tiirismaa Health Center, Hollola	T.Kivelä, L.Petlin, L.Savolainen
Turku Health Center	A.Artukka, I.Hämäläinen, L.Lehtinen, E.Pyysalo, H.Virtamo, M.Viinikkala, M.Vähätalo
Turku University Central Hospital	K.Breitholz, R.Eskola, K.Metsärinne, U.Pietilä, P.Saarinen, R.Tuominen, S.Äyräpää
Vaajakoski Health Center	K.Mäkinen, P.Sopanen
Valkeakoski Regional Hospital	S.Ojanen, E.Valtonen, H.Ylönen, M.Rautiainen, T.Immonen
Vammala Regional Hospital	I.Isomäki, R.Kroneld, L.Mustaniemi, M.Tapiolinna-Mäkelä
Vasa Central Hospital	S.Bergkulla, U.Hautamäki, V-A.Myllyniemi, I.Rusk

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